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The Journal of LABORATORY and CLINICAL MEDICINE

VOL. 42 AUGUST, 1953 NO. 2

The Official Publication of the Central Society for Clinical Research

Clinical and Experimental

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Vol. 42, No. 2, August, 1953. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription price: United States, its possessions, Pan-American Countries, $12.00 per year; Canada, $13.00; Foreign, $13.00. Subscriptions to medical students, interns and residents, half-price. Entered as Second-Class Matter October 8, 1915, at the Post Office at St. Louis, Missouri, under Act of March 3, 1879. Printed in the U. S. A.

The Journal of Laboratory and Clinical Medicine

VOL. 42 AUGUST, 1953 NO. 2

CLINICAL AND EXPERIMENTAL

FACTORS INFLUENCING THE IN VITRO UPTAKE OF RADIOPHOSPHORUS BY HUMAN ERYTHROCYTES

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INTRODUCTION

IT HAS long been known that incubation of human erythrocytes in vitro with tracer quantities of radiophosphorus (as sodium phosphate) results in the incorporation of consistent amounts of radioactivity within the cells.[1, 2] It is also known that certain enzymes concerned in the glycolytic scheme and Krebs cycle, into which the incorporated phosphorus eventually enters,[1, 3, 4] are inactivated by x-radiation.[5] Thus it seemed possible that these phenomena might be used for studying biological effects of ionizing radiation and for screening prospective protective agents—a procedure which now requires extensive survival studies in large numbers of animals. In planning the present experiments it soon became apparent that the usual method for expressing erythrocyte P^{32} uptake as a simple ratio or as percentage of added radioactivity within the red cells would not suffice. The difficulty inherent in such methods of expression can be illustrated by the following two examples:

1. If 1,000 counts per minute of P^{32} are added to 2.0 ml. and also to 4.0 ml. of whole blood (hematocrit 50 per cent), approximately half (500 counts per minute) is taken up by the red cells after two hours of incubation. Thus the percentage uptake in the total erythrocyte mass is the same (50 per cent) in the two samples, but the uptake per unit volume of erythrocytes differs, and is found to be 25 per cent in the first sample and 12.5 per cent in the second.

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Received for publication, Mar. 20, 1953.

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***Work, in part, performed during tenure of a Damon Runyon Senior Clinical Research Fellowship.

2. If 1,000 counts per minute of P^{32} are incubated for two hours with 4.0 ml. of two whole blood samples having hematocrits of 60 and 30 per cent, respectively, the red cells in the first sample will take up twice as many counts per minute as those in the second. However, the percentage uptake per unit volume of red cells is the same in both cases.

The first example demonstrates that with different volumes of the same whole blood different values for P^{32} uptake can be obtained by expressing it as per cent per unit volume of red cells. The second example demonstrates that different values may be obtained with blood of varying hematocrits by expressing the uptake as per cent in total red cell volume. This raises the question of whether the difference is merely a function of concentration, or whether it is due to some intrinsic property of erythrocytes per se. Thus a more discriminating index for evaluating erythrocyte P^{32} uptake is necessary. It should make some provision for the total volume of the suspension in which incubation occurs and for the erythrocyte concentration in that volume. The experiments reported here were first directed toward determination of a simple, consistent expression of P^{32} uptake in erythrocytes, and second toward the variations of such an uptake under different experimental conditions including exposure to ionizing radiation. No attempt was made to determine the uptake of radiophosphorus by red cells as a function of time. Rather the incubation time was kept constant in a region where uptake would be proportional to initial concentration of either P^{32} or cells and where it would vary only with the biochemical integrity of the cells. When uptake of P^{32} by cells is measured as a function of time it has been shown that the problem becomes more complex and that the data can no longer be represented by the simple mathematical relationships presented here.[6]

METHODS

Heparinized venous blood was used, and it was routinely drawn less than one hour before the start of each experimental determination, except as otherwise stated. In some cases whole blood was used, but in others erythrocytes were suspended in saline or glucose-saline before P^{32} was added. It should be noted that red cells which had been washed with saline before resuspension yielded different results from those which were resuspended directly following centrifugation and removal of supernatant plasma by pipetting. In subsequent usage the word "suspension" will be understood to mean erythrocytes suspended in any indicated medium, including plasma.

One microcurie, or less, of carrier-free P^{32} in 0.2 ml. of physiological saline was then added to the red cell suspension. It was gently shaken to obtain homogenous distribution of the P^{32}, and it was incubated at 37.5° C. ± 0.1° for measured periods of time. For brief incubation periods (i.e., less than five hours), manual shaking of the tubes was performed every ten minutes. Any gross deviation from this procedure was found to result in alteration of erythrocyte P^{32} uptake, hence standardization was necessary before another experimental variable could be considered. Even though the volume of red cells (V_r) varied from 0.8 to 4.1 ml. in the experiments performed, the concentration of P^{32} within erythrocytes never became great enough to result in physiological alterations secondary to effects of radiation from tracer. Ten microcuries of P^{32} per milliliter of red cells would have yielded a maximum radiation dose of 40 rep. within the time limits of

incubation. Concentrations used were one-tenth of this or less; and, as will be shown, much greater amounts of radiation have no demonstrable effect upon erythrocyte properties measured here.

After incubation, the suspension was centrifuged at 1100 "G" for fifteen minutes. The total solution volume (V_t) and the volume of red cells (V_i) were recorded. The supernatant was removed and discarded. Physiologic saline in approximately twice the original volume was added, and the mixture was gently agitated. After recentrifugation and removal of the supernatant, two additional saline washings were performed in a similar manner. After the third centrifugation, the final volume of packed red cells (V_f) was noted, and the supernatant again discarded. The packed red cells were then carefully transferred to a 100 ml. volumetric flask and hemolyzed by addition of distilled water to the indicated volume. Aliquots of 2.0 ml. each were prepared in duplicate by slow evaporation to dryness in an aluminum planchet under infrared heat. Appropriately diluted P^{32} standards were prepared, and all samples were counted with a lead-shielded, Geiger-Müller tube (Tracerlab TCG-2). Three saline washings routinely resulted in about 5 per cent reduction of packed red cell volume. Thus V_i is usually about 5 per cent greater than V_f, the difference being presumably lost during the washings.

THEORETICAL CONSIDERATIONS AND DETERMINATION OF AN INDEX FOR P^{32} UPTAKE

The amount of P^{32} taken up by erythrocytes in vitro was noted to be directly influenced by such extrinsic factors as (1) length of time, temperature, and medium in which erythrocytes are preserved prior to P^{32} incubation, and (2) length of time, temperature, and mixing of the suspension during incubation. When the above factors are held constant, the intrinsic factors which affect the erythrocyte P^{32} uptake are the quantity of P^{32} added, the total volume of the suspension, and the volume of the erythroctyes in the suspension. We may conjecture that within the quantitative limits of tracer technique the total uptake in the erythrocytes (U) is directly proportional to the dose of P^{32} added (D) and to the erythrocyte volume (V_f) and inversely proportional to the total volume of the suspension (V_t). Expressed as an equation this becomes

$$U = (K_c)\ (D)\ (V_f) \left(\frac{1}{V_t}\right), \qquad (1)$$

where the symbols represent the measurable quantities noted above and K_c is a proportionality constant. To obtain experimental verification of this equation, the following observations were made.

By algebraic manipulation of its terms, equation (1) may be written in the following three forms:

$$U = \left(K_c \cdot \frac{V_f}{V_t}\right)\ (D) \qquad (2)$$

$$\frac{U}{D} = (K_c \cdot V_f) \left(\frac{1}{V_t}\right) \qquad (3)$$

$$\frac{U}{D} = \left(\frac{K_c}{V_t}\right)\ (V_f) \qquad (4)$$

Equation (2) describes a straight line with slope $\left(K_c \cdot \frac{V_f}{V_t}\right)$, provided $\frac{V_f}{V_t}$ is held constant and the value for D is varied. To plot equation (3), D and V_f are held constant, the volume, V_t, is varied, and U is measured and expressed $\frac{U}{D}$. This equation describes a straight line with slope $(K_c \cdot V_f)$ or it

can represent a hyperbola, depending upon whether the abscissa is plotted in terms of $\frac{1}{V_t}$ or V_t. Equation (4) describes a straight line with slope $(\frac{K_c}{V_t})$, provided D and V_t are held constant, and the variation in U is observed with changes in V_f.

TABLE I. UPTAKE IN RED CELL MASS (U) WITH CHANGES IN DOSE OF P^{32} (D) WHOLE BLOOD; V_f/V_t ESSENTIALLY CONSTANT*

HEMATOCRIT; TIME OF INCUBATION	D (COUNTS/MIN.) $\times 10^3$	V_t (ML.)	V_f (ML.)	U (COUNTS/MIN.) $\times 10^3$	K_c
Sample A 41%; 2 hr.	100	1.9	0.8	65.8	1.56
	200	2.0	0.8	127.6	1.59
	300	2.3	0.9	187.8	1.60
	400	2.0	0.8	251.0	1.57
Sample B 65%; 1 hr.	100	1.9	1.20	61.9	0.98
	124	2.05	1.35	89.0	1.09
	280	1.9	1.20	186.0	1.05
	398	2.0	1.25	263.0	1.06

*Similar results obtained in each case with volumes of whole blood varying from 2.0 ml. to 6.0 ml.

TABLE II. PER CENT UPTAKE IN RED CELL MASS (U/D × 100) VS. TOTAL SOLUTION VOLUME (V_t) AND INVERSE VOLUME ($1/V_t$)

ERYTHROCYTE SUSPENSIONS IN SALINE; V_f KEPT ESSENTIALLY CONSTANT AT 1.0 ML.

1 HR. INCUBATIONS

	V_t (ML.)	$1/V_t$	V_f (ML.)	(U/D) × 100	K_c
A Unwashed cells	3.2	0.312	1.0	36.6	1.175
	4.4	0.227	1.0	28.3	1.244
	5.4	0.185	0.95	24.8	1.311
	6.4	0.156	1.0	19.6	1.255
	7.3	0.137	1.10	17.1	1.250
	11.2	0.089	0.95	12.3	1.375
B Saline-washed cells	2.1	0.476	1.15	28.7	0.524
	2.6	0.385	1.1	23.4	0.552
	3.1	0.323	1.0	20.5	0.634
	3.7	0.270	1.0	17.7	0.652
	4.1	0.244	1.0	16.9	0.694
	5.0	0.200	1.0	13.0	0.581

TABLE III. PER CENT UPTAKE IN RED CELL MASS (U/D × 100) VS. VOLUME OF RED CELL MASS (V_f)

SALINE-WASHED ERYTHROCYTES IN TWO DIFFERENT MEDIA; V_t KEPT ESSENTIALLY CONSTANT AT 4.0 ML.

1 HR. INCUBATIONS

	V_f (ML.)	V_t (ML.)	(U/D) × 100	K_c
A Saline medium	0.25	4.1	4.17	0.684
	0.47	4.1	6.77	0.590
	0.8	4.0	10.5	0.528
	1.3	4.1	17.2	0.545
	1.7	4.1	23.1	0.557
	2.5	4.0	30.8	0.494
B Plasma medium	0.4	4.0	5.4	0.541
	0.95	4.0	11.8	0.498
	1.4	4.1	17.1	0.505
	2.0	4.0	24.0	0.481
	2.5	4.1	33.8	0.554

Numerous experimental determinations of these equations have been made. Sample results are listed in Tables I, II, and III, and the curves drawn in Figs. 1, 2, and 3.

The tabulations and graphs demonstrate, in each case, that the experimental curves obtained were identical with those theoretically predicted by the equations. Moreover, a comparison of the K_c values tabulated for individual points along the curve and those obtained from the slope of the curve

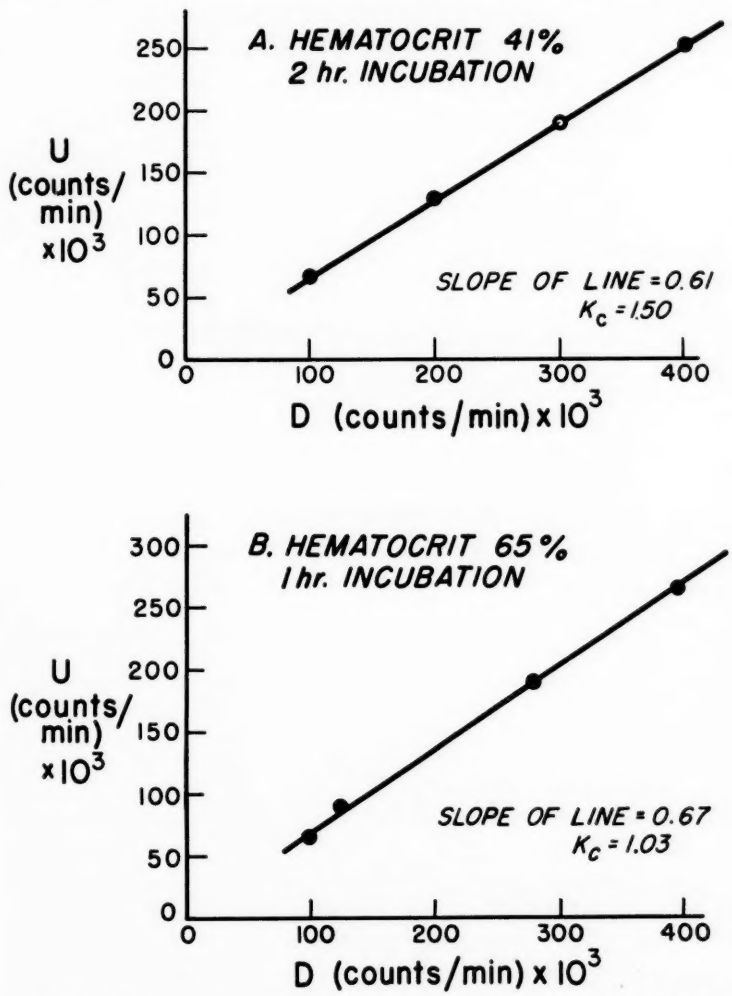

Fig. 1.—P^{32} uptake in red cells (U) vs. dose of P^{32} added (D). Whole blood: V_f/V_t essentially constant.

demonstrates only slight variation. This is consistent with experimental range of error, or with the algebraic compensation necessarily introduced by an occasional curve which does not pass precisely through the origin of the graphical axis. This slight error may be neglected, and routine determination of K_c may be done from individual samples, without necessity for charting the entire curve.

The experimentally established validity of equation (1) thus also establishes the existence of the proportionality constant, K_c. The K_c value will be an individual property of the erythrocytes being tested and may be considered to represent the specific affinity of the red blood cells for P^{32} under standard conditions of incubation. In its final form, K_c may be obtained graphically as demonstrated, or as an individual value derived from the equation

$$K_c = \left(\frac{U}{D}\right)\left(\frac{V_t}{V_f}\right), \qquad (5)$$

where the symbols used are as noted previously, and all terms in equation (5) except K_c are experimentally measured. It can then be used as an index for

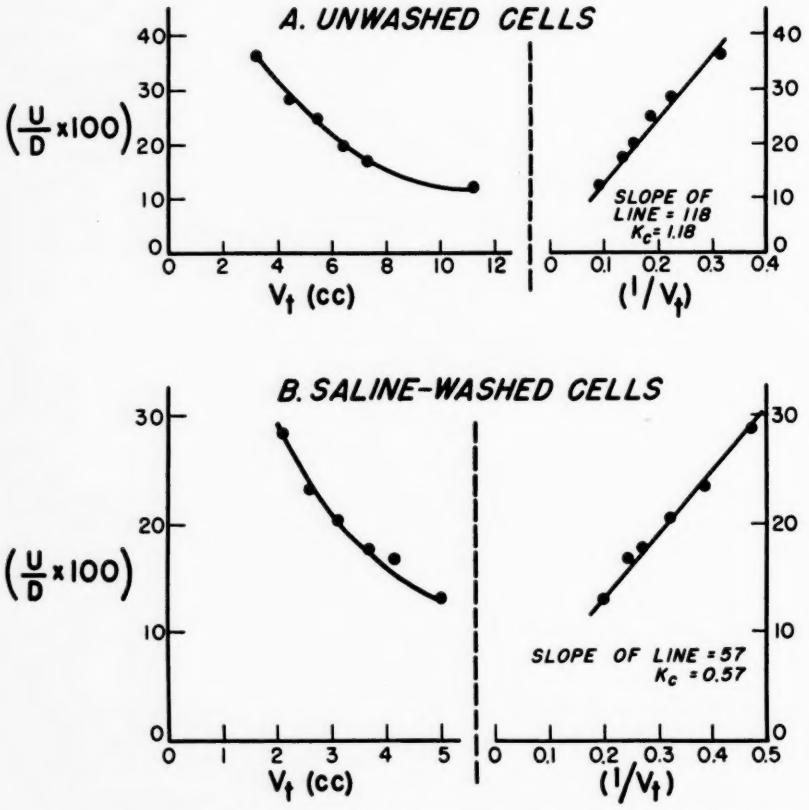

Fig. 2.—Per cent uptake in red cells ($\frac{U}{D} \times 100$) vs. total solution volume (V_t) and inverse volume ($1/V_t$). Saline media; 1 hour incubation; V_f kept constant at 1.0 ml.

evaluating the effect of experimental factors upon erythrocyte P^{32} uptake. This index, it may be seen, makes the desired provision for total suspension volume and for erythrocyte concentration. In its simplest form, when whole blood is the suspension used, equation (5) states that the specific affinity of the red cells for P^{32} is equal, all other factors being constant, to the percentage uptake of P^{32} in the total red cell mass (UD) multiplied by the inverse hematocrit $\left(\frac{V_t}{V_f}\right)$.

Utilizing this criterion of measurement, we have observed the results to be described below.

EFFECT OF EXTRINSIC FACTORS ON K_c

The previous discussion has considered the effect of intrinsic solution factors on erythrocyte P^{32} uptake. Among the extrinsic factors which affect this phenomenon are the temperature, time and mixing of the suspension in which

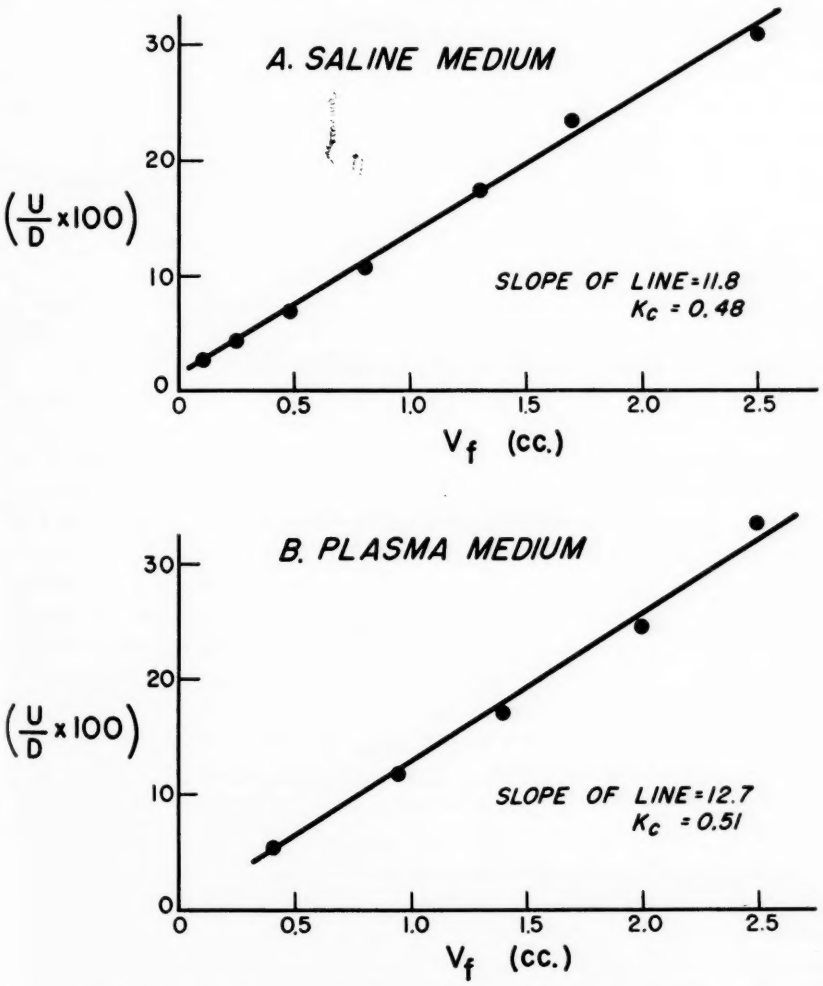

Fig. 3.—Per cent uptake in red cells ($\frac{U}{D} \times 100$) vs. volume of red cells (V_f). Saline-washed cells in two different media; 1 hour incubation; V_t kept constant at 4.0 ml.

incubation with P^{32} is performed. Increased uptake with increasing temperature has been reported by various investigators.[1, 2, 7] Accordingly, all incubations in these experiments were done at constant temperature of 37.5° ± 0.1° C. Increasing uptake with increasing time of incubation has also been ob-

served.[1, 2, 6, 7, 8] In general, the curve of uptake versus time of incubation rises somewhat linearly and then levels off asymptomatically after about two hours. The incubations done in these experiments were usually for measured time intervals of either one or two hours. (In Fig. 1 it may be noted that the K_c for two-hour incubation of whole blood was 1.50, while that for one hour was 1.03.) When the incubation solution is allowed to stand without mixing, a distinct reduction in K_c has been noted. To overcome this phenomenon, a special mixing roller was used by Reeve and Veall,[8] and Gourley and Gemmill[7] have done their incubations in a Warburg apparatus. We have found that manual shaking of the incubated solution performed regularly every ten minutes produces results compatible with those obtained by the more elaborate apparatus, hence the simpler manual method was used.

Other extrinsic factors which affect the specific affinity of the erythrocyte for P^{32} are the medium, temperature, and length of time at which the cells are stored prior to incubation with P^{32}. Some of these factors will be discussed later. There is a distinct difference produced in K_c by the optional process of washing the plasma-deprived cells with saline prior to their instillation in the saline medium for incubation. Fig. 2 demonstrates that higher values are found in the unwashed cells. Yet, as Fig. 3 demonstrates, once the cells have been washed with saline, it makes no difference in their P^{32} uptake whether they are subsequently incubated in autogenous plasma or in saline.

Apparently, all these factors must be considered before the effect of specific experimental conditions upon erythrocyte P^{32} uptake can be evaluated. In the experiments reported below, we have tried whenever possible to keep these constant, or to note wherein they have varied. So long as control suspension tubes were handled in similar manner, their K_c indices could be compared, although different techniques with different groups may result in noncomparable values.

EFFECT OF IRRADIATION ON ERYTHROCYTE P^{32} UPTAKE

A prepared suspension of erythrocytes was exposed to ionizing radiation from a 250 kilovolt, 15 milliampere x-ray machine at a target distance of 12 in., using 0.5 mm. copper and 1 mm. aluminum filters. Total dosages ranged from 20,000 r to 100,000 r, and the dose rate was kept constant. Control suspensions were similarly prepared. K_c determinations were done in some samples immediately following irradiation. In others, the suspensions were stored at 5° C. for measured time intervals and then brought to equilibrium at 37.5° C. before the P^{32} was added for the K_c determination.

Results and Discussion.—The results are listed in Table IV. They demonstrate that x-ray doses of 20,000 to 100,000 r failed to alter significantly the red cell uptake of P^{32}. K_c was determined at various time intervals following irradiation, thus allowing the expected postirradiation hemolysis[9] of some red cells to occur. In solutions in which some hemolysis occurred, however, the surviving cells preserved the same specific affinity for P^{32} as the nonirradiated cells. A recent study by Berlin and associates[10] suggested that there were two

types of red cells present in polycythemic blood. The present results might be interpreted to suggest that normal blood contains two types of circulating erythrocytes, one fraction which is easily hemolyzed by irradiation, and another which is quite resistant to irradiation and which preserves its enzyme systems. Another interpretation of these results is that since circulating erythrocytes are normally a mixed population of various ages, irradiation increases the decay process and thus causes hemolysis in all cells above a critical age level, while it has no effect upon cells whose age is below the critical value.

TABLE IV. EFFECT OF IRRADIATION UPON K_c AT VARIOUS POSTIRRADIATION INTERVALS

PREPARATION OF ERYTHROCYTES	DOSE IN ROENTGENS	TIME OF P^{32} INCUBATION (HR.)	INTERVAL BETWEEN IRRADIATION AND INCUBATION (HR.)	K_c OF IRRADIATED SAMPLES	K_c OF CONTROL SAMPLES
Heparinized whole blood	30,000	2	1	1.61	1.54
	100,000	1.8	1	1.20	1.08
				1.24	1.33
	20,000	1	23	0.641	0.658
				0.692	0.713
	20,000	1	47	0.661	0.667
				0.539	0.622
	20,000	1	72	0.323	0.458
				0.457	0.502
	20,000	1	96	0.342	0.355
Saline-washed cells in saline medium	30,000	1	4	0.503	0.591
	30,000	1	21	0.531	0.557
	30,000	1	25	0.498	0.508
	30,000	1	45	0.495	0.583

K_c VARIATION IN DISEASE

The correlation of K_c with clinical disease conditions was next considered. Whole heparinized blood was drawn from several normal donors and from twenty patients hospitalized with various clinical disorders. The blood was allowed to stand overnight at 5° C. and then brought to equilibrium at 37.5° C. for a two-hour incubation and K_c determination. The following results were obtained:

The results in Table V have a mean value, for these experimental conditions, of 0.471 for the twenty-four samples tested, with a standard deviation of 0.04. In cases where a significant difference from the mean was thought to have occurred, repeated examination of K_c in these individuals produced values within the normal range and, in some, values deviating from the mean in the opposite direction. Thus the numerical results do not follow a normal distribution pattern, nor is there any consistent correlation between the type of clinical condition and K_c. These results, therefore, do not show, in this small sample, any indication that the K_c index might be clinically useful as a diagnostic or therapeutic criterion. Pertzoff and Gemmill[11] studied normal blood donors and found considerable variation in P^{32} uptake among different donors, and diurnal variations among samples from an individual donor. These variations were as great or greater than those found in the diseases just listed. They have not reported studies done in pathologic conditions.

TABLE V. VARIATION OF K_c WITH CLINICAL DISEASE CONDITIONS IN TWENTY-FOUR INDIVIDUAL CASES

DIAGNOSIS	K_c
Normal	0.469
Normal	0.447
Normal	0.532
Normal	0.473
Lipodystrophy	0.414
Cushing's syndrome	0.487
Diabetes mellitus	0.479
Hyperthyroidism	0.411
Arteriosclerotic congestive heart failure	0.506
Essential hypertension	0.428
Essential hypertension	0.478
Kimmelstiel-Wilson syndrome*	0.406
Chronic glomerulonephritis*	0.454
Iron deficiency anemia	0.434
Acquired hemolytic anemia	0.424
Subacute bacterial endocarditis	0.445
Duodenal ulcer	0.561
Duodenal ulcer	0.480
Gastric ulcer	0.467
Ulcerative colitis	0.420
Carcinoma of prostate	0.550
Carcinoma of pancreas	0.540
Carcinoma of breast	0.444
Reactive depression	0.545

*Not in uremia.

EFFECT OF ERYTHROCYTE STORAGE ON K_c

The alterations which occur in stored red blood cells have been the subject of numerous investigations,[12-18] many of which have pointed out the vital role played by glucose in the metabolic survival of the erythrocyte. Moreover, the incorporation of P^{32} into the red cell has been shown to be ultimately an enzymatic process in which glucose again plays an important role.[1, 3, 7] An excellent review of erythrocyte enzyme systems has been written by Bartlett and Marlow.[3] Hence, the presence of glucose in the storage medium might be expected to affect the survival and thus the enzymatic integrity of stored erythrocytes. Since K_c is a measure of P^{32} uptake and thus of enzymatic integrity, its use in evaluating the effects of storage upon red cells was proposed in the experiments reported below. These experiments were in no sense planned to perform a thorough investigation of the effects of storage phenomena upon erythrocytes. Rather, they were planned as a preliminary guide in indicating that the effects of storage were reflected in changes in the K_c index. Further experiments to correlate changes in K_c values with other methods used to assess red cell viability and integrity may demonstrate that the simply performed K_c measurement is as sensitive as some of the more complex methods used to evaluate these phenomena.

Methods.—Heparinized whole blood was centrifuged to obtain packed erythrocytes. One milliliter of the unwashed cells was transferred to 15 ml. centrifuge tubes into which had previously been added 3.0 ml. of 0.85 per cent saline containing various amounts of glucose. The final glucose concentration was calculated on the basis of a total solution volume of 4.0 ml. The suspensions were stored for various time intervals and at specific temperatures. At the end of

that time, each suspension, regardless of its storage temperature, was brought to 37.5° C., P^{32} was added, and it was incubated for two hours at 37.5° C. The erythrocyte K_c was then determined. In solutions in which partial hemolysis had occurred, there was no change in the previously described procedure, and the P^{32} uptake was counted in those cells remaining after the usual three saline washings. The K_c value was thus a test of the specific P^{32} affinity of those cells which survived the storage.

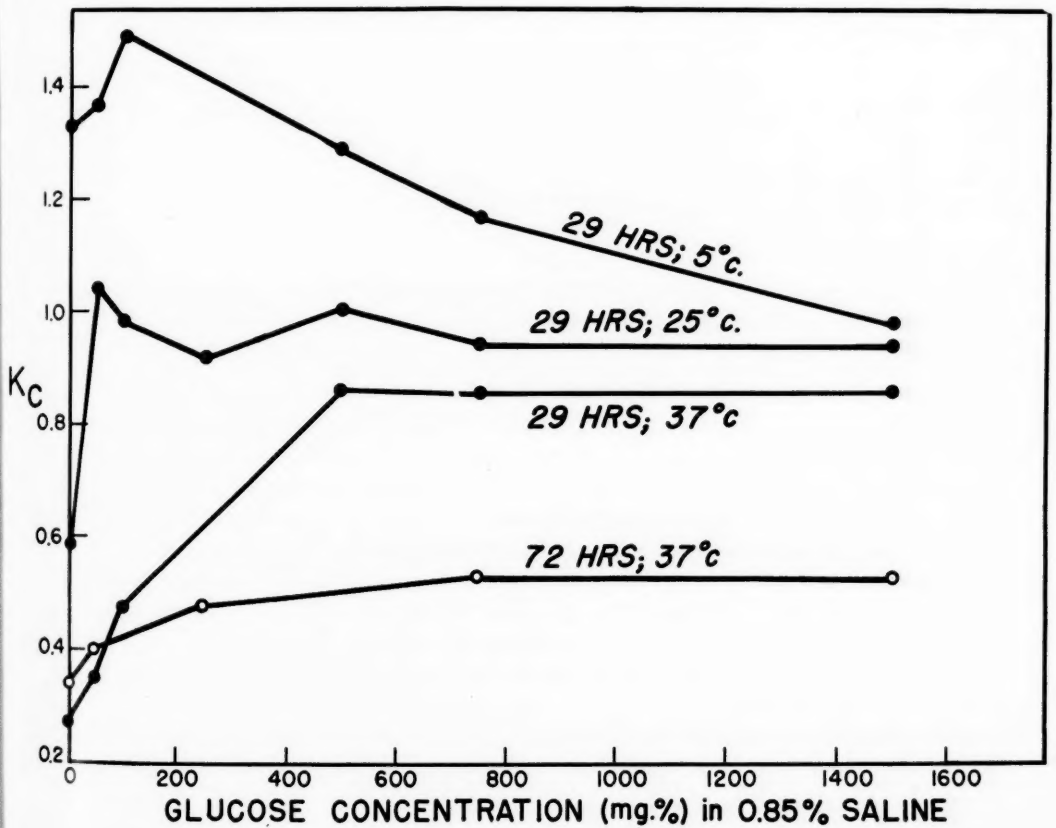

Fig. 4.—Effect of certain storage conditions on K_c. Unwashed cells in saline media with various concentrations of glucose; stored at different temperatures, and for different times; all P^{32} incubations done for two hours at 37.5° C.

Results.—Curves for K_c variation with glucose concentrations at different temperatures and times of storage have been plotted in Fig. 4. No studies were carried on beyond a seventy-two hour storage period. At a temperature of 37° C. and after twenty-nine hours of storage, the K_c index is about 0.85 for all concentrations of glucose above 500 mg. per cent, and is considerably below this value for glucose concentrations less than 100 mg. per cent. When the cells are allowed to stand at this temperature for seventy-two hours, the entire curve drops to a K_c value considerably below the previous ones. At room temperature

(25° C.) and after twenty-nine hours, the K_c curve is almost flat, indicating that glucose has little effect on cell preservation under these conditions. For this same time at 5° C., the values obtained are considerably higher with low or no glucose than with the higher concentrations up to 1500 mg. per cent. It should be noted that all three curves for the twenty-nine hour storage period and different temperatures approach identical K_c values with increasing glucose concentrations.

These data suggest that the K_c value may parallel the metabolic or respiratory rate as determined in a Warburg apparatus, and thus may be a dependable estimate of cellular viability. If so, use of this simple, brief procedure could greatly facilitate assessment of the efficiency of various methods of blood preservation. Expression of results as K_c rather than percentage uptake automatically corrects for differences in hematocrit in samples tested and permits interlaboratory comparison, provided extrinsic conditions (time of incubation, temperature, mixing, etc.) are standardized.

SUMMARY

A new index for expressing P^{32} uptake by erythrocytes has been developed and it is determined from the following experimentally established equation:

$$K_c = \left(\frac{U}{D}\right)\left(\frac{V_t}{V_f}\right),$$

where U is the uptake in the red cell mass, D is the incubated dose of P^{32}, V_t is the total suspension volume, and V_f is the volume of the packed red cells.

This new index, K_c, represents the specific affinity of the red cell for P^{32} under standard conditions of incubation, and it makes provision for the effects of hematocrit and total solution volume upon erythrocyte P^{32} uptake.

Irradiation has been shown to have little or no effect on the human erythrocyte uptake of P^{32} and, hence, this method is not suitable for use as an in vitro screening technique for proposed anti-irradiation agents.

Erythrocyte K_c values show no consistent variation over a wide range of clinical disease conditions.

Storage of red cells with varying amounts of glucose at different times and temperatures suggests that the K_c of the surviving cells reflects their viability. It is proposed that this might be a simple, useful tool in the search for more effective methods of blood preservation.

The authors wish to express their appreciation to Dr. E. S. G. Barron, Dr. A. M. Brues, Dr. L. O. Jacobson, and Dr. J. M. Goldinger for their kind advice and counsel.

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ERYTHROCYTE CARBOHYDRATE METABOLISM

I. The Flow of C^{14}-Glucose Carbon into Lactic Acid, Carbon Dioxide, Cell Polymers, and Carbohydrate Intermediate Pool

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CARBOHYDRATE metabolism in the mature mammalian erythrocyte consists chiefly of a glycolytic split of glucose to lactic acid. Early studies were reviewed by Lundsgaard.[1] Past investigations have emphasized the properties and variations of acid-soluble organic phosphorus. Guest and Rapoport[2] have summarized this field, and Granick[3] has included a discussion of metabolism in his review on the chemistry of the erythrocyte.

Information is still fragmentary on the exact nature of the carbohydrate intermediates and their possible function in the metabolism of normal and abnormal cell types. The availability of C^{14}-tagged glucose has made it possible to explore more extensively the pathway taken by glucose carbon in its metabolism by the intact surviving human red cell. This paper evaluates the use of glucose carbon by the erythrocytes for the formation of lactic acid, carbon dioxide, cell polymers, and the pool of metabolic intermediates. This carbohydrate intermediate pool has been divided into a number of barium fractions which have been analyzed for phosphorus, glucose, fructose, adenine, triose, pentose, and pyridine nucleotide, and from these analyses a preliminary estimation of the amount of a number of known carbohydrate intermediates and coenzymes has been made.

METHODS

Four large-scale experiments were carried out, the amounts being chosen to provide sufficient material for isolation of trace constituents. Blood was obtained from young normal donors (Table I).

To 492 ml. of defibrinated blood was added 8 ml. of a solution containing 160 mg. of uniformly labeled C^{14}-glucose, and the mixture was incubated four hours in air at 38° C. with gentle shaking. Following the incubation the cells were washed three times at the centrifuge with ice cold isotonic saline, the surface coat of leukocytes and fat particles

Table I. Characteristics of Experimental Bloods

Blood	Sex	Age	Hematocrit (per cent)	Hemoglobin (gm.)	Reticulocytes (per cent)	R.B.C. $\times 10^{-6}$	W.B.C. $\times 10^{-3}$
1	F	35	46	15.0	0.8	5.11	7.5
2	M	25	48	15.2	0.6	5.20	5.7
3	M	30	48	15.2	0.5	5.05	6.4
4	F	36	43	14.6	0.5	4.65	5.9

From The Scripps Metabolic Clinic, La Jolla, Calif.

This investigation was supported in part by research grant 1071-C from the National Heart Institute of the National Institutes of Health, Public Health Service.

Received for publication, Feb. 16, 1953.

being carefully aspirated and discarded. The washed cells were extracted with trichloroacetic acid (TCA). The TCA was removed with ether and the extract dried by lyophilization.

Glucose and lactic acid were purified from the combined serum and cell-wash fluids after deproteinization with perchloric acid. The lactic acid was extracted with ether, purified as the zinc salt, and oxidized with permanganate-sulfuric acid to acetaldehyde,[4] which was purified as the 2,4-dinitrophenylhydrazone. Glucose was isolated as the phenylglucosazone.

The trichloroacetic acid soluble material was made to 50 ml. with water, and barium-insoluble, alcohol-insoluble, and alcohol-soluble fractions made according to the procedure of LePage.[5] A small amount of the original material was now water insoluble and was set aside and labeled the water-insoluble fraction. Likewise a portion of the barium-insoluble fraction would not redissolve in acid. This was labeled the acid-insoluble fraction.

Separate 25 ml. incubations were used for collecting carbon dioxide. Lactic acid was added following the incubation and CO_2 free air drawn through the mixture into 30 ml. of 0.3N sodium hydroxide for a period of one hour.

For carbon and C^{14} radioactivity assay, appropriate amounts of the various fractions were oxidized by wet combustion,[6] and the carbon dioxide was counted as barium carbonate.

The following analytical methods were employed: glucose, copper-arsenomolybdate, Nelson[7]; lactic acid, acetaldehyde-hydroxydiphenyl, Barker and Summerson[8]; fructose, resorcinol, Roe[9]; pentose, orcinol, Mejbaum[10]; triose, alkaline hydrolysis, Lohmann and Meyerhof[11]; phophorus, ammonium molybdate-aminonaphtholsulfonic acid, Fiske and Subbarow[12]; total phosphorus digestion, sulfuric acid-hydrogen peroxide; pyridine nucleotide, acetone-fluorimetric, Levitas and associates[13]; adenine, E 260 mμ Beckman DU.

RESULTS AND DISCUSSION

Table II summarizes the distribution of metabolized glucose in various fractions of the red blood cell. Since all carbon atoms of the sugar are equally labeled, the product of the carbon content and specific activity of a fraction divided by the specific activity of the glucose being used gives the number of carbons derived from glucose.

Experiment 2 will be used as an example for discussion of the results. The initial glucose content of the blood was 565 mg. including 160 mg. of C^{14} glucose containing 56,000 counts per milligram of carbon. Assuming complete mixing, the total glucose would have an activity of 15,900. Of the 565 mg. of glucose initially present, 236 mg. was metabolized. A portion of the remainder was isolated in pure form as the phenylosazone and counted after oxidation to barium carbonate. The activity corrected for the inert carbons in the molecule was 15,000. The figures in the table represent the glucosazone calculated values.

The initial lactic acid was 57 mg., 238 mg. additional being formed during the incubation, a quantity equivalent to the glucose disappearing. To determine the activity of the lactic acid, it was isolated from the cell-wash fluid at the end of the experiment, purified as the acetaldehyde-2,4-dinitrophenylhydrazone and counted as carbonate. Corrected for the inert carbon of the derivative and for the lactic acid present at the start of the incubation, the formed lactic acid had an activity of 7,500. Ninety-four milligrams of glucose

TABLE II. DISTRIBUTION OF GLUCOSE CARBON AFTER FOUR HOURS OF METABOLISM AT 38° C. BY 500 ML. OF BLOOD

EXPERIMENT	MG. MATERIAL				MG. CARBON				TOTAL COUNTS × 10^{-3}				COUNTS/MG. CARBON			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Glucose used	202	236	242	232	81	94	97	93	1190	1410	1440	1350	14700	15000	14800	14500
Lactic formed	234	238	216	197	94	95	86	79	680	710	620	600	7200	7500	7200	7600
CO_2									9.0	9.5	8.8	9.2				
TCA insoluble	78g	82g	92g	84g	38g	40g	44g	40g	0	0	0	0				
TCA soluble	4.2g	4.9g	4.0g	4.0g	305	305	295	280	610	700	660	640	2000	2300	2250	2270
Barium insoluble	654	543	633	628	74	66	72	71	530	500	520	520	7100	7500	7250	7350
Alcohol insoluble	284	243	249	221	81	75	75	73	69	83	71	66	850	1100	950	900
Alcohol soluble	5.4g	5.6g	4.3g	5.2g	120	115	110	115	48	52	50	48	400	450	450	420
Water insoluble			41	46			10	8			25	20			2500	2500
Acid insoluble	50	82	71	45			5	3			8	5			1500	1500

g equals grams.

carbon was used and 95 mg. of lactic acid carbon formed. Since the specific activities were respectively 15,000 and 7,500, 1,410,000 counts of glucose were utilized and 710,000 counts, or, in other words, 50 per cent of the glucose carbons appeared in the lactic acid. The remainder of the lactic acid was derived from non-tagged components of the cell carbohydrate permanent pool. This pool in turn was tagged by that glucose used which did not appear in lactic acid.

We are next concerned with the fate of the glucose carbons which disappeared but did not reach lactic acid. Three sites were examined, carbon dioxide and the trichloroacetic acid insoluble and soluble portions of the saline washed cells.

Due to the large amounts of carbon dioxide normally present in the blood, about 27 mg. of carbon per 100 ml., it is not possible to determine accurately whether there is any real net increase during the incubation period. However, the radioactivity of the total carbon dioxide at the end of the experiment shows exactly how many glucose carbons were oxidized to carbon dioxide. An aliquot of the blood was incubated for four hours following which all the carbon dioxide was collected and radioassayed. Corrected to the total blood volume, 9,500 counts were found which represent only 0.7 per cent of the total glucose metabolized or 0.7 mg. of CO_2 carbon newly formed from glucose.

It is necessary to consider possible contributions to carbohydrate metabolism of blood components other than the erythrocyte. Of the cell population in the experimental bloods, there were approximately 0.1 per cent leukocytes and 0.5 per cent reticulocytes. Almost all of the platelets appear to have been removed during the process of defibrination. There was no evidence that the cell-free serum could, by itself, carry out any metabolism of glucose, although there was some indication that it supported a somewhat higher and more stable rate of glycolysis in the cells than did salt solutions of appropriate tonicity.

Reticulocytes[14] and leukocytes[15] show a measurable respiration in contrast to the mature erythrocyte which appears to consume little or no oxygen. There is insufficient information for an accurate evaluation of the respiratory contribution of reticulocyte and leukocyte under the conditions (large excess of erythrocytes) of our experiments. A rough estimate, nevertheless, can be made, the values chosen probably being on the high side. If the QCO_2 (from glucose) (microliters of carbon dioxide per milligram dry weight per hour) of the reticulocyte is 1, it can be calculated that this would supply approximately 0.6 mg. of CO_2 carbon in 500 ml. of blood in four hours at 38° C. If the average white cell QCO_2 (from glucose) is 5, then the white cell respiration would give about the same amount of CO_2. Our yield of 0.7 mg. of CO_2 carbon is considerably under this estimated sum and could be entirely due to these contaminants. It seems likely that the erythrocyte contributed little or nothing to CO_2 formation.

It has been reported that the white cells possess a relatively high aerobic lactic acid formation from glucose.[15] Assuming a value Q lactic acid (air) = 10, 0.1 per cent white cells in 500 ml. of blood in four hours would produce about 3.5 mg. of lactic acid carbon or 3.7 per cent of the total lactic acid formed in the experiment. This then could contribute slightly to the glucose utilization and lactic acid formation in the blood. Approximately 90 per cent of the white cells were removed by aspirating the buffy coat at the time of the centrifugal washing of the cells. Therefore, they had an insignificant influence on the factors found in the red cell TCA extracts.

Following the incubation period, the washed red cells were divided into trichloroacetic acid insoluble and soluble parts. The TCA insolubles had less than 10,000 total counts or, in other words, less than 1 per cent of the glucose carbon utilized. The large amount of carbon (from hemoglobin) prevents an accurate assay of traces of activity. However, the negative results prove that the mature red cell possesses an insignificant apparatus for the synthesis from glucose of protein or TCA insoluble lipid and polysaccharide.

The trichloroacetic acid soluble portion of the red cell contains most of the glucose carbon which did not reach lactic acid. This fraction is composed of the inorganic salts and small molecule organic constituents of the cell, including all of the carbohydrate intermediates. It has 305 mg. of carbon out of about 41,000 mg. total in the cells. If 95 per cent of the cell carbon is hemoglobin, then the TCA soluble is made up of approximately 15 per cent of the non-hemoglobin carbon. The remainder includes such components as the enzymes catalyzing sugar breakdown, catalase, cholinesterase, phosphatase, peptidase, glutathione, and stroma substance.

The 305 mg. of carbon in the TCA solubles had an activity of 2,300. Since the glucose being metabolized was 15,000, 15.3 per cent or 47 mg. of the TCA carbon was derived directly from the glucose. This contained 700,000 counts or 50 per cent of the 1.41 million utilized. This tagged carbon constitutes a pool of intermediates along the pathway of glucose metabolism, and the next problem is to determine the nature of the compounds involved.

As a first step toward identifying these compounds the total extract was divided into a number of fractions depending on the solubility of the barium salts.[5] The three major fractions were barium-insoluble, alcohol-insoluble and alcohol-soluble.

The barium-insoluble fraction had 66 mg. of carbon containing 500,000 counts, approximately three-fourths of the total radioactivity in all fractions. The activity of 7,500 shows that 50 per cent of its carbons were derived directly from the sugar being metabolized. The alcohol insoluble had 75 mg. of carbon and was much less active, being only 7.3 per cent labeled. The alcohol solubles contained 115 mg. of carbon, 3 per cent labeled in respect to the glucose being used. A portion of the alcohol-soluble activity is due to traces of free glucose and lactic acid which were not washed from the cells.

Two minor components were isolated which are not usually represented in carbohydrate metabolism studies, water-insoluble and acid-insoluble fractions. The water-insoluble fraction was a brownish residue left when the

lyophilized TCA extract was dissolved in water. It was originally soluble in the two liters of extract (TCA removed) before drying. After repeated washings, it maintains a relatively high radioactivity and, therefore, is a glucose derivative.

The acid-soluble fraction likewise does not conform with any expected intermediate. It precipitates with the barium-insoluble fraction, but does not redissolve on repeated extraction with strong HCl. This product also maintains a high radioactivity and so must be some glucose metabolite. It is, of course, possible that either of these minor fractions may represent derived products modified by the experimental manipulations.

To identify further the individual parts of the carbohydrate pool, the barium fractions were analyzed for phosphorus, glucose, fructose, adenine,

TABLE III. ANALYSES OF BARIUM FRACTIONS FROM TRICHLOROACETIC ACID-SOLUBLE FRACTIONS OF HUMAN ERYTHROCYTES

	MICROMOLES PER 100 ML. OF RED BLOOD CELLS		
ANALYSIS	BARIUM-INSOLUBLE	ALCOHOL-INSOLUBLE	ALCOHOL-SOLUBLE
Glucose	0	39	50
Lactic acid	0	0	44
Fructose	0	6.7	4.4
Ribose	102	44	15
Adenine	89	53	15
Triose	0	1	0
DPN	0	3.5	6.2
Total carbon	2630	2820	4260
Carbon from glucose	1300	182	124

TABLE IV. PHOSPHORUS IN THE BARIUM FRACTIONS FROM TRICHLOROACETIC ACID-SOLUBLE FRACTIONS OF HUMAN ERYTHROCYTES

	MICROMOLES INORGANIC PHOSPHORUS FORMED PER 100 ML. OF RED BLOOD CELLS		
TIME OF HYDROLYSIS IN 1N HCL AT 100° C.	BARIUM-INSOLUBLE	ALCOHOL-INSOLUBLE	ALCOHOL-SOLUBLE
0	43	4.2	0
10 Minutes	236	45	2.2
1 Hour	255	67	5.3
3 Hours	400	78	13
Total, after digestion in H_2SO_4–H_2O_2	1290	133	20

TABLE V. ESTIMATED CONTENT OF INDIVIDUAL COMPOUNDS IN BARIUM FRACTIONS FROM TRICHLOROACETIC ACID SOLUBLES OF HUMAN ERYTHROCYTES

COMPOUND	BARIUM-INSOLUBLE	COMPOUND	ALCOHOL-INSOLUBLE	ALCOHOL-SOLUBLE
Adenosine triphosphate from ribose	102	Glucose-1-phosphate	6	2
Adenosine triphosphate from 10 min. P	93	Glucose-6-phosphate	50	?
Adenosine triphosphate from adenine	89	Fructose-6-phosphate	10	7
Diphosphoglycerate	407	Adenosine monophosphate	37	9
Hexose diphosphate	0	Diphosphopyridineucleotide	3.5	6.2
		Triose phosphate	1	0

Figures represent micromoles of compound in fraction from 100 ml. of red blood cells.

triose, pentose, and pyridine nucleotide (Tables III and IV), and from these analyses a preliminary estimation of the amounts of a number of known carbohydrate intermediates and coenzymes were made (Table V). The figures represent the averages of the four blood runs, maximum variation being plus or minus 10 per cent. The two minor fractions, water-insoluble and acid-insoluble, have not yet been examined in detail.

All values are calculated for 100 ml. of red cells. In the barium-insoluble fraction there were 2,630 microatoms of carbon, one-half of which originated from the glucose utilized during the four-hour incubation. The other two fractions had somewhat more total carbon, but much less derived from glucose. For comparison total phosphorus was predominant in the barium insolubles with 1,290 microatoms; the alcohol insolubles and alcohol solubles containing, respectively, only 133 and 20 microatoms.

A large part of the barium-insoluble fraction is composed of ATP and DPG, present in a molar ratio of approximately 1 to 4. These two overshadow in quantity all other compounds estimated in the three fractions. The evidence for the ATP comes from the close equivalence in the expected molecular ratio of adenine to ribose to ten-minute hydrolyzable phosphorus. However, in every analysis the ribose value was a little high, suggesting the presence of small amounts of some other pentose derivative.

There are no good specific tests for glycerate, and the DPG is calculated on the assumption that it corresponds to the total phosphorus minus the three-hour P minus 40 per cent of the stable P of adenosine triphosphate. That most of the acid resistant phosphorus is truly DPG is indicated from a number of studies of blood DPG[2] including isolation of the pure compound.[16, 17] Small amounts of other compounds, such as monophosphoglycerate, would not be disclosed by these methods.

Ninety-one micromoles of ATP contain 910 microatoms of carbon and 273 microatoms of phosphorus. Four hundred seven micromoles of DPG give 1,221 microatoms of carbon and 814 microatoms of phosphorus. The two together supplied 2,131 microatoms of carbon and 1,087 microatoms of phosphorus, 81 and 84 per cent, respectively, of the totals.

Hexose monophosphates, adenosine monophosphate, and pyridine-nucleotide are the principal estimated components of the alcohol-insoluble fraction. There is a variable carry-over of these compounds into the alcohol solubles. Negative qualitative tests for pyruvate on one-hour hydrolyzed samples indicated insignificant amounts of phosphopyruvate. A small positive reaction was obtained by the alkaline phosphorus test for triose (phosphoglyceraldehyde and dihydroxyacetone phosphate) which was confirmed by lactic acid analysis of the hydrolytic product.

An appreciable amount of DPN was found, as has been shown by others.[13] The fluorescent method would include triphosphopyridine-nucleotide and N-substituted nicotinamide split products of these coenzymes, although DPN appears to be the principal nicotinamide derivative of tissue extracts.

Adenosine-5-monophosphate was computed on the premise that it corresponds to that ribose in excess of the 2 moles required per mole of DPN. Cor-

rected for the amount in DPN, an equivalent of adenine should also be present, but this substance was found about 35 per cent high.

Glucose-1-phosphate is not reducing as such but, after ten minutes acid hydrolysis, gives theoretical glucose and inorganic phosphorus values. Adenylic acid also hydrolyzes in ten minutes to ribose-5-phosphate, 1 mole of which gives a sugar test equivalent to one-half mole of glucose. A ten-minute hydrolysis increase of glucose of 25 μM was found in the alcohol-insoluble fraction. The amount of AMP estimated would provide 10 μM of "glucose," leaving only 6 μM which could be furnished by GlP. The ten-minute phosphorus increase was 41, whereas only 6 would come from GlP. There are indications that the fraction is contaminated with some ATP which would add to the ten-minute phosphorus, but this source could not make up the difference. Therefore, an unexpected labile ester should be sought in this fraction.

Glucose-6-phosphate is the major hexosemonophosphate present in the alcohol insolubles. It is calculated from the glucose value of the unhydrolyzed extract corrected for the reducing power of fructose-6-phosphate. All fructose found is assumed to be present as the 6-phosphate.

The compounds listed in the alcohol insolubles account for 111 microatoms of phosphorus of the total of 133 microatoms found. They add up to 843 microatoms of carbon, only 30 per cent of the total in the fraction. Therefore, there remains a small fraction of unknown phosphorus and a large amount of unidentified carbon, most of which is not labeled and so not directly associated with the carbohydrate intermediates. The phosphorus hydrolysis rate found was consistent with known rates of the tabulated compounds.

In the alcohol-soluble fraction there is some free glucose and lactic acid which were not washed from the cells after the incubation. They could not be removed quantitatively because of the necessity of working rapidly to prevent breakdown of the phosphate esters. It is likely that some other compounds contribute to the values reported, in view of the low radioactivity of the fraction. These analyses were not too satisfactory because of the large amounts of inorganic salts present.

The ten-minute P of the alcohol solubles is calculated as GlP. All fructose is considered to be F6P. G6P, although probably carried over into this fraction, was not estimated because of interfering reducing material. A sizable amount of adenine and ribose was found in equivalent quantities and is labeled AMP, although adenosine or other purine-pentose derivatives could be responsible. A majority of the total DPN appeared in this fraction and not in the expected alcohol insolubles even though, during the preparation, volumes were kept at a minimum and a long period in the cold allowed for precipitation. The fraction contains very little phosphorus and a large amount of unknown carbon of little or no radioactivity.

As summarized in the review by Guest and Rapoport,[2] there have been a number of reports on "hexose phosphates," organic pyrophosphate, and diphosphoglycerate in the red blood cells. The present investigation represents a more extensive analysis of carbohydrate intermediates in deproteinized acid extracts of erythrocytes. Recently Gourley,[18] in connection with studies on phosphorus permeability in human erythrocytes, using different analytical

and fractionation methods, has also estimated some carbohydrate intermediates. Only with DPG[16, 17] and ATP[19, 20] have the individual compounds been isolated and identified, and even with ATP the structure is assumed to be the usual adenine-ribose-5-triphosphate. Further fractionation with varied and more specific methods of analysis is necessary positively and quantitatively to establish these substances and to disclose others which undoubtedly are present in trace amounts.

SUMMARY

After a period of incubation with C^{14}-labeled glucose, human red blood cells were fractionated to determine the pathways of metabolism and the nature of the cell permanent pool of carbohydrate intermediates. Although lactic acid was formed equivalent in quantity to the glucose used, the time of incubation was such that only 50 per cent of the lactic acid carbon was derived from glucose, the remainder coming from the nonlabeled carbohydrate storage mixture of the cell which, in turn, was replaced by labeled carbons from glucose. This pool of tagged metabolic intermediates amounted to 20 mg. of carbon per 100 ml. of cells, approximately 15 per cent of the total trichloroacetic acid soluble carbon and about 0.1 per cent of the total cell carbon.

The TCA soluble mixture was divided into five fractions as a preliminary to analysis and isolation of the individual compounds, and the carbon content and specific activity of each determined. Three-fourths of the activity was found in the barium insolubles which contained about one-fourth of the TCA soluble carbon.

Essentially negative activity appeared in the TCA insolubles emphasizing the red cell's lack of capacity for protein or fat synthesis.

Slightly less than 1 per cent of the glucose carbon went to carbon dioxide, and it was calculated that this was probably due to contaminating reticulocytes and leukocytes rather than demonstrating any real respiratory capacity of the mature red cell.

The barium-insoluble, alcohol-insoluble, and alcohol-soluble fractions were assayed for glucose, lactic acid, fructose, ribose, adenine, triose, total carbon, carbon derived from metabolic glucose, total phosphorus, inorganic phosphorus, and organic phosphorus hydrolyzed at ten minutes, one hour, and three hours. These values give a picture of the chemistry of the fraction from which an estimate of the following individual compounds was made: adenosine triphosphate, diphosphoglycerate, fructose diphosphate, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, adenosine-5-phosphate, diphosphopyridinenucleotide, and triose phosphate. The estimates and unexplained deviations which have been noted provide a chart for further exploration of the exact nature of carbohydrate intermediates and related substances.

Cooperation of the San Diego Blood Bank is gratefully acknowledged.

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ERYTHROCYTE CARBOHYDRATE METABOLISM

II. Chromatographic Isolation of Monophosphoglycerate, Diphosphoglycerate, and Adenosine Triphosphate and Their Metabolic Turnover with Glucose Carbon

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WE HAVE been concerned with the trichloroacetic acid (TCA) extractable components of the human erythrocyte, in particular those which can be related to carbohydrate metabolism.[1] Per 100 ml. of red blood cells there were found 11 millimoles of organic TCA soluble carbon, 1.7 mM of which was replaced by C^{14}-glucose carbon during a four-hour incubation at 38° C. This acid-soluble carbon containing a labeled pool of carbohydrate intermediates was divided into fractions depending on the solubilities of the barium salts. These were then subjected to a variety of analyses from which the content of a number of known carbohydrate intermediates and coenzymes was estimated.

The barium insolubles from 100 ml. of red cells had 2.6 mM of carbon, 50 per cent being derived from metabolized glucose. Analyses indicated that the fraction contained 1.2 mM of diphosphoglycerate carbon and 0.9 mM of adenosine triphosphate carbon. This communication describes a separation of the barium insoluble compounds by ion exchange chromatography and demonstrates by radioassay the extent of their participation as intermediates in glucose breakdown.

METHODS

The components of the red cell barium insolubles have been resolved by ion-exchange chromatography, the technique and principles being essentially the same as described by Cohn.[2]

Dowex-1, 200-400 mesh (Dow Chemical Co.), an anion exchanger, was washed several times with distilled water, removing fine and some large particles by decantation. An aqueous slurry of the resin was poured into a tube containing a coarse sintered plate so that on settling the volume of the resin was 1 sq. cm. by 6 cm. The column was prepared by washing with 100 ml. of 1 N HCl followed by water until the effluent was chloride negative. The sample was absorbed on the column and differentially removed with various eluting agents. Using a flow rate of 2.5 ml. per minute, approximately 10 ml. fractions were collected with an automatic fraction collector (Microchemical Specialties Co.).

Approximately 40 mg. of the barium insolubles from the blood experiments previously described[1] was dissolved in 2 ml. of 1N HCl in a 12 ml. centrifuge tube and heated for twenty minutes at 100° C. in a steam bath. The cooled solution was diluted to 8 ml., and 1 ml. of 5 per cent Na_2SO_4 added. The barium sulfate was centrifuged down and

From The Scripps Metabolic Clinic.
This investigation was supported in part by research grant 1071-C from the National Heart Institute of the National Institutes of Health, Public Health Service.
Received for publication, Feb. 16, 1953.

washed twice with 5 ml. portions of water. The supernatant and washings were neutralized to phenol red with 1N NaOH and diluted to 100 milliliters. Two milliliters of 1N NH_4OH was added to insure slight alkalinity and uptake by the resin of the weakly absorbed adenine. After washing with about 100 ml. of water, the following series of eluting agents were used: 400 ml. of 0.001N HCl, 400 ml. of 0.003N HCl, 300 ml. of 0.01N HCl in 0.01N NaCl and 300 ml. of 0.01N HCl in 0.1N NaCl.

The distribution of compounds in the fractions was determined by analysis of adenine, ribose, and inorganic and organic phosphorus, and confirmed by comparing the elution pattern of authentic compounds. The materials and analytical methods have been described.[1] 3-monophosphoglycerate was obtained from Schwarz Laboratories, Inc.

Radioactivity was measured by evaporating 0.5 to 1.0 ml. aliquots on planchets. The counts on resulting "thin layer" planchets could be directly compared with one another because total solid was less than 1 mg. per square centimeter, a region giving a relatively small self-absorption error.

RESULTS AND DISCUSSION

There are two reasons for seeking a complete isolation of each component of the TCA soluble extract: (1) for more certain identification, and (2) to assign accurately the C^{14} radioactivity distribution. It will be seen that the ion exchange method allows for a complete separation and quantitative recovery of the principal components of the blood barium insolubles.

We have been interested especially in determining the quantitative radioactivity level of diphosphoglycerate and also in investigating the possibility of any flow of glucose carbon into the adenine or ribose parts of adenosine triphosphate. Since even a small carbon shunt into ribose would be of great interest, this compound had to be completely rid of any trace of the highly active DPG.

During the course of this study, numerous attempts were made to separate ATP and DPG. In brief, it can be stated that ATP could be removed completely from DPG, but the reverse was extremely difficult. Mercuric salt precipitation left DPG free of nucleotide, but significant amounts of DPG coprecipitate with the HgATP. Some charcoals absorbed all nucleotide from a solution of the mixture, but the ATP on the carbon was contaminated with DPG. Twenty-minute hydrolysis of the mixture followed by barium salt fractionation[3] gave adenine entirely in the alcohol soluble, and phosphoglycerates in pure state on reprecipitation of the barium-insoluble fraction. However, ribose-5-phosphate in the alcohol-insoluble fraction continued to be contaminated with DPG after several reprecipitations. Lack of recognition of this contaminating effect of the highly labeled DPG early led us to the erroneous conclusion that ribose had a relatively strong radioactivity. These observations emphasize the deficiencies of the usual salt fractionation purification techniques involving, in addition, large losses of yield due to partial solubilities in precipitating solutions and wash fluids.

Even the Dowex-1 resin did not readily distinguish ATP and DPG. They both eluted together at a concentration of 0.01N HCl-0.1N NaCl, or a concentration of 0.06N HCl. Slight variations in the acidity or salt content of the eluting fluid did not result in significant separation.

Fortunately for our purposes we are not so much interested in recovering the intact ATP molecule as the individual carbon entities, ribose and adenine.

During biosynthesis of ATP it is most probable that the adenine and ribose portions are made separately and then combined. ATP completely hydrolyses in ten to twenty minutes to give adenine, ribose-5-phosphate, and inorganic phosphate. During this hydrolysis period mono- and diphosphoglycerates are unaffected. A mixture of pure ATP, 3-monophosphoglycerate, and 2,3-diphosphoglycerate were hydrolyzed for twenty minutes at 100° C. in 1N HCl. The resulting adenine, ribose-5-phosphate, MPG, and DPG were found to be satisfactorily resolved by the resin column.

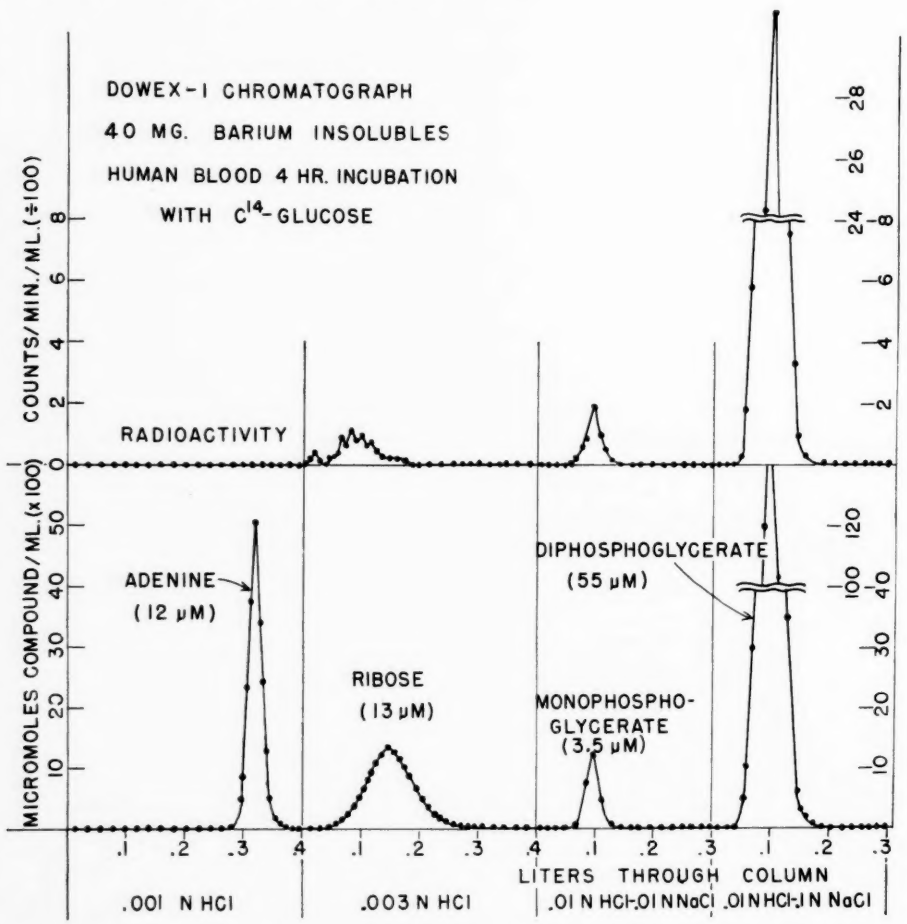

Fig. 1.—Ion-exchange chromatographic separation of the components of the barium insoluble fraction of a trichloroacetic acid extract of human erythrocytes which had been incubated with C^{14}-glucose for four hours at 38° C.

The figure summarizes the results of the ion-exchange chromatography of 40 mg. of barium insolubles from the No. 3 blood run. Aliquots of the barium insolubles of the other three blood experiments gave comparable results. Adenine came off the column at 0.001N HCl, ribose-5-phosphate at 0.003N HCl, MPG at 0.01N HCl-0.01N NaCl, and DPG at 0.01 HCl-0.1 NaCl. The elution

position in the case of each component exactly coincided with that obtained from a mixture of pure ATP, MPG, and DPG treated in an identical manner. The curves represent 260 mμ absorption values for adenine, orcinol test for ribose, and total organic phosphate for MPG and DPG (in both cases found to be resistant to three-hour hydrolysis at 100° C. in 1N HCl). A 24 μM increase of inorganic phosphorus appeared in the 0.003 HCl fraction, added confirmation for the hydrolytic cleavage of ATP to one equivalent each of ribose and adenine and two equivalents of inorganic phosphate. The total amounts recovered were 12 μM of adenine, 13 μM of ribose-5-phosphate, 3.5 μM of MPG, and 55 μM of DPG.

The radioactivity curve shows quantitatively which compounds have picked up glucose carbon during the red cell metabolism. There was a high peak of radioactivity exactly in the same position as the DPG and a small peak coinciding with the MPG. Each of the phosphoglycerates, on the basis of the calculated carbon content, had a "thin layer planchet" activity of about 60,000 counts per milligram of carbon. Due to the absorption of the soft betas from C^{14} the corresponding "saturated planchet" value would be about 12,000 counts per milligram of carbon, only slightly less than the sugar being metabolized.

A small amount of radioactivity appeared in the 0.003 fraction. Its peak precedes slightly the center of the ribose elution and so there is doubt as to how much, if any, is due to the ribose. The irregular nature of the radioactivity in this area indicates the possible presence of more than one trace component. It could be that a small amount of hexose phosphates—the bulk of which appear in the alcohol insolubles—are contaminating this fraction. We have found that mixed hexose monophosphates elute at approximately this position.

It has already been demonstrated with P^{32} that hydrolysis resistant organic acid soluble phosphorus of the red cell has a high rate of turnover.[4, 5] In this paper we have established the fact that a very large per cent of the phosphoglycerate carbon is renewed from glucose during a four-hour period. Renewal of ribose carbon, if any, is quite small. There was no trace of radioactivity associated with the adenine peak of the chromatograph, showing that none of this compound could have been synthesized from glucose.

From past investigations there has been little doubt that ATP[6] and DPG[7] were present in the red cell in relatively large quantities. The analysis presented here offers a more satisfactory separation and isolation of these constituents, and of the MPG, in terms of their carbon parts, and has enabled us to assess their participation as intermediates in glucose breakdown. The aim is to integrate these values into as complete a picture as possible of the erythrocyte carbohydrate metabolism. Research is in progress to complete the identification of the carbohydrate pool, in order eventually to have a red cell carbohydrate intermediate spectrum for comparison in various physiologic and pathologic states.

SUMMARY

The carbon constituents of the barium-insoluble fraction of trichloroacetic acid extracts of human erythrocytes have been isolated by ion exchange chromatography as adenine, ribose-5-phosphate, 3-monophosphoglycerate and 2,3-diphosphoglycerate. The phosphoglycerates showed a high turnover rate with glucose carbon, adenine none, and ribose-5-phosphate possibly a trace.

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STUDIES ON ABNORMAL HEMOGLOBINS

VII. The Composition of the Non-S Hemoglobin Fraction in Sickle-Cell Anemia Bloods. A Comparative Quantitative Study by the Methods of Electrophoresis and Alkali Denaturation

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Chicago, Ill.

IT IS now well established that stroma-free hemolysates prepared from bloods of patients with typical sickle-cell anemia often reveal on electrophoretic analysis not only the presence of S (sickle-cell) hemoglobin, but also of a second component in amounts of from 5 to 20 per cent.[1, 2] This latter component has a mobility slightly slower than that of S hemoglobin, but identical with that of normal adult (type A*) hemoglobin under the conditions of electrophoresis most suitable for the separation of the S compound. It has, furthermore, been noted that, under these same experimental conditions, fetal (type F) hemoglobin has the same mobility as the A pigment.[2, 10]

F hemoglobin can be distinguished readily from A hemoglobin by its increased resistance to alkali denaturation.[3] It has been demonstrated in this laboratory by means of a special alkali denaturation technique[4] that blood specimens from patients with typical sickle-cell anemia almost regularly contain fetal hemoglobin in quantities varying from 2 to 24 per cent.

In order to decide whether the non-S hemoglobin fraction of sickle-cell anemia hemolysates is solely composed of the alkali-resistant compound, or contains a mixture of A and F hemoglobin, a comparative study of such hemoglobin solutions by these two methods seemed to be indicated. Should the amounts of F hemoglobin, as obtained by the alkali denaturation technique, agree with the electrophoretic measurements of the non-S hemoglobin fractions, it would be possible to express the S hemoglobin content of such hemolysates as the difference between the total hemoglobin concentration and the percentage of alkali-resistant pigment. Should, however, no good agreement between these two results be found, and should the non-S hemoglobin electrophoresis values be significantly higher than those obtained with the chemical method, the presence in the solution of type A hemoglobin, besides the F compound, would have to be assumed.

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The Department is supported in part by the Michael Reese Research Foundation. This work was carried out with the aid of a grant from the United States Public Health Service and was also supported by the Hulda B. and Maurice L. Rothschild Foundation for Scientific Research.

Received for publication, Feb. 20, 1953.

*In previous contributions of this series the letter N denoted normal adult hemoglobin. At a recent symposium on abnormal hemoglobins,[5] it was suggested that the following symbols be used: A for normal adult, F for fetal, S for sickle-cell, and C for type III[6, 7] hemoglobins. D was proposed for a rare type of abnormal hemoglobin which moves electrophoretically like S hemoglobin, but does not cause sickling when present in erythrocytes.[8]

MATERIAL AND METHODS

Only bloods with alkali denaturation values of 5 per cent or more, obtained from patients with typical sickle-cell anemia, were used in this study. This selection was made because of the difficulty encountered in accurately separating by electrophoresis less than 5 per cent of a second component when the mobility of the latter is very close to that of the major component.[9] The comparative determinations were carried out on the same blood sample from each patient, since it has been observed that small daily variations of the values for F hemoglobin may occur in the same individual.[4] Specimens containing type C hemoglobin[6, 7] were excluded. It has been our experience, gained from the study of 11 patients, that only small amounts of F hemoglobin may be found in this variant of sickle-cell disease.[10]

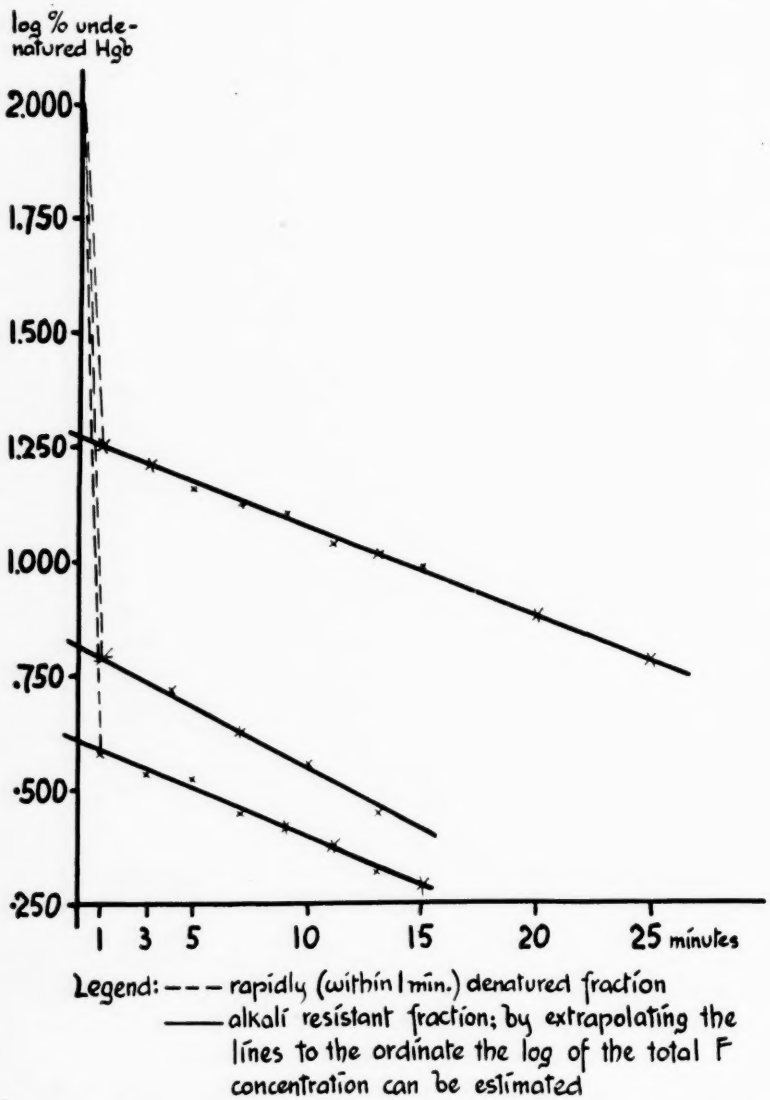
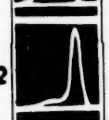
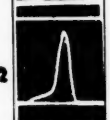
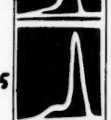
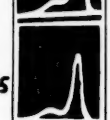
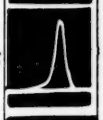
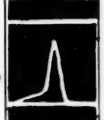


Fig. 1.—Fractional denaturation of three sickle-cell anemia hemolysates.

The electrophoretic analyses were performed in a Tiselius apparatus (American Instrument Company), utilizing a cylindrical lens-diagonal slit optical system. The hemoglobin solutions were first prepared in a manner identical with that used for the alkali denaturation procedure,[4] but were diluted to 1 per cent with a cacodylate-sodium chloride buffer of pH 6.5 and 0.1 ionic strength. The pigment was converted to carboxyhemoglobin, and dialyzed against a large volume of buffer. Electrophoresis was carried out in a stand-

Specimen No.	% Total Alkali Resistant Hgb: Oxy Compounds	Carboxy Compounds	Electrophoresis Values % Hgb: Non-S	S	Pattern*	Difference betw. Electrophoresis & Denatur. Values: Oxy Compounds	Carboxy Compounds
1	18.8	18.2	18.8	81.2		0	0.6
2	18.7	19.9	17.8	82.2		0.9	2.1
3	17.0	18.2	18.3	81.7		1.3	0.1
4	15.1	16.4	14.0	86.0		1.1	2.4
5a	11.0	15.0	16.8	83.2		5.8	1.8
5b	10.6	13.6	5.8	94.2		4.8	7.8
6	8.4	10.0	8.8	91.2		0.4	1.2
7	7.0	9.4	10.0	90.0		3.0	0.6
8	14.6	15.2	17.5	82.5		2.9	2.3
9	20.0	21.8	19.5	80.5		0.5	2.3

No.	% Total Alkali Resistant Hgb: Oxy Compounds	Carboxy Compounds	Electrophoresis Values % Hgb: Non-S	S	Pattern*	Difference betw. Electrophoresis & Denat. Values: Oxy Compounds	Carboxy Compounds
10	11.6	14.2	18.5	81.5		6.9	4.3
11	4.1	5.1	7.5	92.5		3.4	2.4
12	4.2	5.8	8.8	91.2		4.6	3.0
13	8.3	10.2	13.6	86.4		5.3	3.4
14	5.5	7.2	9.4	90.6		3.9	2.2
15	6.4	8.6	10.7	89.3		4.3	2.1
16	11.8	12.5	11.4	88.6		0.4	1.1

Standard Deviation

	Oxy-F Hgb	Carboxy-F Hgb
σ	2.19	1.76
3σ	6.57	5.28

*) The electrophoretic migration proceeds from left to right

Fig. 2.—Comparison of the values of the non-S hemoglobin fraction in sickle-cell anemia as determined by alkali denaturation and electrophoresis.

ard Tiselius cell at 1.5 degree Celsius, under a potential gradient of 5.3 volts per centimeter. Only the ascending boundaries were used for interpretation. Planimetric measurements of the various components were made according to the method of Tiselius and Kabat.[11]

The total amount of F hemoglobin in the hemolysates was determined by the method of fractional denaturation.[12] With this procedure, the progress of the denaturation process of F hemoglobin is followed by permitting the alkali reagent to react with identical portions of the same hemoglobin solution for intervals of increasing lengths of time, and determining the remaining amounts of the unaffected pigment in these samples. It should be emphasized that with the alkaline denaturation technique, as used in this laboratory, types A, S, and C hemoglobin are almost completely destroyed within one minute.[4, 10] By carrying out the fractional denaturation procedure after one, three, five, ten, fifteen, and twenty-five minutes, it is possible to distinguish in nearly all sickle-cell anemia hemolysates two pigment fractions: one which is completely destroyed within one minute, and another which is gradually denatured. When the logarithms of the percentages of the unaffected hemoglobin concentrations, found at varying intervals, are plotted against time in a diagram, a straight "line of disappearance" of the resistant pigment is readily demonstrable.[12] This indicates that a single type of abnormally resistant pigment is present.[13] By extrapolating this straight line to the ordinate, the total amount of the F component may then be estimated[13] (Fig. 1).

Helpern and Strassman[14] have pointed out that normal adult carboxyhemoglobin reveals a slightly increased resistance to alkali denaturation as compared with the oxy-compound. We have been able to confirm this observation, and have also noticed that the denaturation values of the F carboxyhemoglobin are slightly higher than those of the F oxypigment. Since the electrophoretic analyses were carried out with carboxyhemoglobin solutions, it was decided to determine the total amounts of F hemoglobin by means of the use of the oxy- as well as the carboxycompounds.

RESULTS

I. Comparison of the Quantitative Determinations, by Electrophoresis and Alkali Denaturation, of the Non-S Hemoglobin Fraction in Sickle-Cell Anemia Hemolysates.—Fig. 2 shows the results of such a comparative study with the hemolysates prepared from the bloods of 16 patients with classical sickle-cell anemia. As can be seen, the values for F hemoglobin obtained with the carboxycompounds are, in 15 of 16 instances, higher than those for the oxypigments. In many previous experiments we have found that the maximal range of deviation from the average alkali denaturation value may amount to ±8 per cent when ten successive determinations are performed on the same specimen. On this basis it may be stated that at least some of the denaturation values of the carboxyhemoglobins are definitely greater than those found with the use of the oxypigments. Why this discrepancy is more pronounced in some specimens (Fig. 2, *5a* and *5b**) than in others is not clear.

In general, the results of the determinations with the electrophoretic and the alkali denaturation techniques were in excellent agreement. The standard deviation (σ) of the differences between the values for the non-S hemoglobin fraction as obtained by these two unrelated methods amounted to 1.76 for the carboxy- and to 2.19 for the oxyhemoglobin compounds. Since the series of observations was relatively small, the statistically required correction for

*Because of the relatively marked difference in the results of the alkali denaturation technique for the oxy- and carboxy compounds in specimen *5a*, the comparative studies were repeated in a second blood sample (*5b*) of the same patient.

small series was applied to the calculation of the standard deviation.[15] Using 3 σ (Fig. 2) as the "level of significance,"[15] any value outside 3 σ may either indicate some error in the performance of the tests, or the presence of another component in the non-S fraction of the hemoglobin solutions. In only one instance (Fig. 2, *5b*) was the difference between the values obtained by the two methods significantly outside the 3 σ range. In this particular experiment,

Exp. No.	Specimen: Mixture % Cord Hemoglobin	Specimen: Mixture % S	Composition % Hgb: F	A	S	% Alkali Resistant (F) Hgb: Expected	Found	% A Hgb (by difference) x)	Electrophoresis Values % Hgb: Non-S	S	Pattern
1	100	0	67.0	33.0	0	-	67.0	33.0	100.0	0	
2	75	25	50.3	24.7	25	50.3	51.0	22.1	73.1	26.9	
3	50	50	33.5	16.5	50	33.5	37.0	15.0	52.0	48.0	
4	25	75	16.8	8.2	75	16.8	18.2	1.4	19.6	80.4	

Standard Deviation: σ 1.76
(for the carboxy compound) 3σ 5.28

x) electrophoresis value minus alkali denaturation value

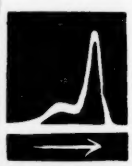
Fig. 3.—Comparison of the values as obtained by electrophoresis and alkali denaturation for the non-S hemoglobin fraction in artifical mixtures containing A, F, and S hemoglobin.

however, the electrophoresis value was lower than that found by alkali denaturation. This may possibly signify a methodical error in the electrophoresis value.

II. Comparison of the Quantitative Determinations, by Electrophoresis and Alkali Denaturation, of the non-S Hemoglobin Fraction in Artificial Mixtures Containing A, F, and S Hemoglobin.—The results of the experiments described above seem to suggest (1) that normal adult (type A) hemoglobin is entirely

absent in the hemolysates prepared from bloods of patients with sickle-cell anemia, and (2) that, therefore, the amount of S hemoglobin can simply be expressed as the difference between the total hemoglobin and the alkali-resistant pigment.

To test the validity of this conclusion, artificial mixtures of a cord blood hemoglobin solution—containing both the A and F pigments—and a S hemoglobin solution were prepared and examined by both methods. The S hemoglobin solution was obtained from one of the few patients we have encountered, whose blood had almost no F hemoglobin (less than 2 per cent). Since in these artificial mixtures the non-S hemoglobin fraction included the readily

Pat. No.	% Total Alkali Resistant Hgb		Electrophoresis			Difference betw. Electrophoresis & Denat. Values	
	Oxy Compounds	Carboxy Compounds	Values % Hgb Non-S	S	Pattern	Oxy Compounds	Carboxy Compounds
1	11.3	13.3	25.5	74.5		14.2	12.2
2	11.7	12.9	29.5	70.5		17.8	16.6

Standard Deviation:

	Oxy Hgb	Carboxy Hgb
σ	2.19	1.76
3σ	6.57	5.28

Fig. 4.—Comparison of the electrophoresis and alkali denaturation values in hemolysates containing S, F, and A hemoglobin.

denatured A pigment in addition to the alkali-resistant F compound, the electrophoresis value for this fraction should, theoretically, always be greater than that found by the denaturation procedure. The results of these experiments are presented in Fig. 3.

The cord blood hemoglobin solution contained 67 per cent of F and 33 per cent of A hemoglobin. Electrophoretically, this mixture appeared as a single component (Fig. 3, *experiment 1*). When three parts of the cord blood hemolysate were mixed with one part of the S hemoglobin solution (Fig. 3, *experiment 2*), the non-S hemoglobin fraction totaled 73.1 per cent. There was excellent agreement between the calculated and the actually demonstrated values for the F pigment. When the mixture contained *equal* parts of the two hemoglobin solutions (Fig. 3, *experiment 3*), the electrophoresis results for the non-S

pigment fraction was 52 per cent. The figure for the alkali-resistant hemoglobin actually found was 10 per cent higher than expected. It must be considered that this aberration may be, in part, due to the small amount of alkali-resistant material present in the S hemoglobin solution, which was omitted from the calculation of the expected result for the purpose of simpler presentation.

When one part of the hemolysate containing A and F hemoglobin was mixed with three parts of the S hemoglobin solution (Fig. 3, *experiment 4*), the non-S hemoglobin fraction, as visualized electrophoretically, fell 5.4 per cent short of its theoretical value. This deviation lies within the often encountered range of variations of the electrophoretic method. The difference between the results for the non-S fraction in this particular experiment, as determined by both techniques, was not more than 1.4 per cent, therefore apparently in excellent accord. However, closer inspection shows an obvious discrepancy between the calculated results and those obtained. Because the actual electrophoresis value was lower, and the alkali denaturation value somewhat higher than expected, the amount of A hemoglobin (8.2 per cent) could not be detected. This finding indicates that even when there is apparently close agreement between the results with both techniques—which was also observed with the sickle-cell anemia hemolysates—the presence of a small quantity of A hemoglobin cannot be ruled out definitely. This uncertainty regarding the presence of small amounts of A pigment in sickle-cell anemia hemolysates is attributable to the relative insensitivity of both methods employed.

III. Results Obtained by the Comparative Study of the Hemolysates from Two Exceptional Cases.—During the course of our studies, we encountered two hemolysates whose electrophoresis values for the non-S hemoglobin fraction were markedly higher than the results obtained by alkali denaturation. The data of these two exceptional instances are compiled in Fig. 4.

The first patient (Fig. 4, No. *1*), a 40-year-old Negro woman, is the mother of a 10-year-old child with typical sickle-cell anemia. It may be mentioned that this child had the highest F value (24 per cent) ever observed in our laboratory.[4] The patient's hemoglobin was 13.2 Gm., with 4.4 million erythrocytes. Her reticulocyte count fluctuated between 1.5 and 2.8 per cent, in the absence of any gross bleeding. The erythrocytes sickled readily with the sodium metabisulfite method, and there was a moderate number of target cells in the stained film. The osmotic fragility of her red cells was decreased, with complete hemolysis at 0.16 per cent sodium chloride. The erythrocytic-granulocytic ratio of her marrow was 1:2. On numerous occasions, this patient had F hemoglobin values varying from 10.3 to 13.4 per cent. Since it has been shown, in almost 200 carriers of the sickle-cell trait, that no F hemoglobin is demonstrable in their bloods although A and S hemoglobin are regularly found,[4, 10] this patient cannot be classified as a typical sickle-cell trait. Furthermore, her S hemoglobin amounted to 74.5 per cent of her total hemoglobin which is much more than the percentage for S encountered in the usual sickle-cell trait bloods.[1, 10] In our series[10] of 40 individuals with the trait anomaly, the S pigment varied from 20.3 to 50.0 per cent of the total hemoglobin, which

confirms the estimates of Wells and Itano[1] who give figures from 22 to 45 per cent. No C hemoglobin was demonstrable in this hemolysate. Whether the S hemoglobin fraction also contained D hemoglobin cannot be stated. Type D hemoglobin moves electrophoretically like S hemoglobin, but is more soluble in buffered solutions, and can thus be distinguished by appropriate solubility studies.[2, 8] This approach was not known at the time when our experiments were performed. However, as can be seen from Fig. 4, No. *1*, this patient's blood contained also a definite quantity of A hemoglobin (12.2 to 14.2 per cent), since the non-S hemoglobin fraction amounted to 25.5 per cent by electrophoresis, whereas the alkali denaturation values were only about one-half of this figure. Because the difference between these results with the two unrelated techniques is considerably outside the 3 σ range, it must be assumed that in this particular hemolysate not only S and F, but also A hemoglobin are present.

A relatively high electrophoresis value for the non-S fraction, only partly accounted for by F hemoglobin, was also noted in a blood sample (Fig. 4, No. *2*) from a 15-month-old Negro girl sent to our laboratory from the Charity Hospital, New Orleans, La.* This patient had been born prematurely, had shown poor development, and had been underweight since birth. She was admitted with an upper respiratory infection and hepato-splenomegaly. The sickling test was positive, and some sickled erythrocytes were seen in the film. There was a normochromic normocytic anemia (Hb. 5.6 Gm., R.B.C. 1.8 million, W.B.C. 8,700, M.C.V. 100 cubic microns). There were 160 nucleated red cells per 100 leukocytes, and the reticulocyte count amounted to 39 per cent. The hypotonic saline fragility was decreased, complete hemolysis occurring at 0.18 per cent sodium chloride. The Coombs test was negative. X-ray films of the skull and long bones revealed cortical thinning and increased cortical markings.

Electrophoretic analysis showed 70.5 per cent S hemoglobin and 29.5 per cent of a slower-moving component. The F hemoglobin value of the carboxy compound was 12.9 per cent. The difference between the results of the two methods thus amounted to 16.6 per cent, which is considerably outside of the range of 3 σ. This finding, therefore, again denotes the presence of type A hemoglobin. Special inquiries showed that no blood transfusion had apparently been given four months prior to the analysis of the specimen. Since the anemia was of the normocytic normochromic type, microdrepanocytic disease (sickle-cell disease plus hereditary leptocytosis[16, 18, 22]) can probably be excluded. Unfortunately, no follow-up studies were possible.

DISCUSSION

Two kinds of normal human hemoglobin, the adult (A) and the fetal (F), and three abnormal types named S, C, and D have so far been identified.[5] It has been demonstrated that the alkali-resistant F hemoglobin, present at birth in concentrations of from 50 to 85 per cent,[4, 17] may sometimes require about

*We are indebted to Dr. Philip Pizzolato for this specimen, and for supplying the clinical and laboratory findings.

TABLE I. "DIFFERENTIAL" HEMOGLOBIN ANALYSIS OF THE VARIOUS SICKLING DISORDERS

SICKLING DISORDER	"DIFFERENTIAL" HEMOGLOBIN ANALYSIS						REMARKS	HEMATOLOGIC FEATURES			
	MAJOR COMPONENT		MINOR COMPONENTS								
	TYPE	%	TYPE	%	TYPE	%		ANEMIA	RETICULOCYTE COUNT	SICKLING IN FILM	TARGET CELLS
Sickle-cell trait[10*]	A	50-80	S	20-50	—	—	F Hb. always absent with alkali denaturation technique	absent	normal	absent	absent
Sickle-cell anemia[10*]	S	76-100	F	2-24	A	?	(1) presence of A Hb. in small amounts possible (see text) (2) 3 (i.e., 3.4%) out of 87 bloods examined contained no F Hb.*	marked (normochromic normocytic)	markedly elevated	present	present
C variant[7, 10*]	C	50-67	S	33-48	F	2-3	F Hb. may be absent		slightly to moderately elevated	absent	present (numerous)
D variant (rare)†	D	?	S	?	F	?	D Hb. may be absent	–	–	–	–
Microdrepanocytic disease[10,* 16, 18, 22]	S	60*-70	A	23-33*	F	7-17*	simultaneous expression of sickling and hereditary leptocytosis genes	hypochromic microcytic	may be marked	absent or present	present

*Data based on own observations.
†These data are entirely taken from Itano's papers.[2, 8] We have not yet identified hemolysates with D Hb.

thirty months to disappear from the blood of normal infants.[17]* Beyond this age group, any demonstrable abnormal quantity of alkali-resistant pigment is a significant indicator of a severe disturbance in erythrocyte production.[4] S hemoglobin is the only type of pigment which gives rise to the sickling phenomenon of erythrocytes. It is only rarely found without being accompanied by any of the other hemoglobins (Table I). According to the company it keeps, and its quantitative representation, the "differential" hemoglobin analysis of such hemolysates permits a more comprehensive classification of the various types of sickle-cell disorders (Table I).

The electrophoretic technique which led to the discovery of the abnormal hemoglobins remains, for the time being, still indispensable for any "differential" hemoglobin studies. It is now established that under the conditions most suitable for the electrophoretic separation of S hemoglobin (pH 6.5), this pigment has a mobility intermediate to that of A and F hemoglobin, which move identically but slower, and to C hemoglobin, which moves faster.[6, 7] D hemoglobin has the same mobility as S hemoglobin, but does not produce sickling.[8] To avoid erroneous interpretations of electrophoretic patterns any hemolysate must, therefore, also be investigated by the alkali denaturation technique and, when the presence of D hemoglobin is suspected, by appropriate solubility studies.[8] Before the demonstration of F hemoglobin in nearly all sickle-cell anemia bloods,[4, 23] the non-S hemoglobin fraction of the latter was believed to be entirely composed of A hemoglobin.[1]

Quantitative determinations by different authors of this non-S hemoglobin fraction have shown values up to 20 per cent by electrophoresis,[1] and up to 24 per cent by alkali denaturation.[4] Because of this agreement it was assumed[2, 4]—but never proved—that this fraction was solely composed of the alkali-resistant compound. To ascertain whether this assumption was correct, the amounts of total F hemoglobin, as determined by fractional denaturation, were compared in this study with the electrophoretic estimations of the non-S hemoglobin fraction in hemolysates obtained from 16 patients with classical sickle-cell anemia. In almost all instances a close accord of the results was found, apparently indicating the complete absence of A hemoglobin in such hemolysates.

However, when mixtures of A, F, and S hemoglobin were examined with both techniques, it was noted that an amount of 8.2 per cent A hemoglobin escaped detection; in this particular experiment the alkali denaturation value was somewhat higher, and the electrophoresis result somewhat lower than calculated, although the values actually found by both methods were in close agreement with each other. Thus, small quantities of A pigment may not be demonstrable in a hemolysate due to the relative insensitivity of the techniques employed. From that it follows that sickle-cell anemia hemolysates possibly could also contain some A hemoglobin, despite the apparently excellent accord of the comparative results. On the other hand, it must be considered unlikely

*Chernoff has recently presented evidence (J. LAB. & CLIN. MED. **40**: 787, 1952) which indicates that the bloods of the great majority, if not all, of normal adults contain smaller amounts of fetal hemoglobin (less than 1 per cent) when tested by means of an immunologic technique. This finding would indicate that minute amounts of fetal hemoglobin, not detectable by the alkali denaturation method, may still be present in a hemolysate.

that in the investigations of the 16 hemolysates the same technical aberrations occurred constantly which were responsible for the nondetection of the 8.2 per cent of A hemoglobin in the artificial mixture. Therefore, it may be concluded that the amount of A hemoglobin in sickle-cell anemia hemolysates—if present at all—must be very small, and for practical purposes its possible presence may be ignored. Thus it seems permissible to express the quantity of S hemoglobin in such hemolysates according to the equation:

per cent S Hb. = total Hb. concentration (100 per cent) − per cent F Hb.

This is of significance to laboratories which are not equipped to perform electrophoresis, but can apply the alkali denaturation technique which is inexpensive, rapid, and simple. However, it should be realized that occasionally the above given equation may not be entirely valid and that such exceptions can be identified only by means of electrophoresis. Such atypical cases may be suspected from the careful analysis of the clinical and hematologic features (Table I).

For the two exceptional hemolysates with a relatively large non-S hemoglobin fraction only partly accounted for by the quantity of F pigment demonstrable, the "differential" hemoglobin analysis points to the existence of microdrepanocytic disease[18] (Table I). However, the clinical data militate against this diagnosis since the first patient was not anemic at all, and the second had a normochromic normocytic anemia, apparently not masked by any transfusions, with an extremely high reticulocyte count. Whether the blood of the first patient contained D hemoglobin cannot be stated since this type of hemoglobin was not yet discovered when the specimen was examined. In the second patient, the low S hemoglobin percentage and the unusually high reticulocyte count are definitely atypical. Unfortunately, follow-up studies were not feasible in either instance. We record these exceptional results to emphasize the necessity for many more fact-finding investigations of the various sickling disorders.

It has also been reported in the literature that type A hemoglobin may be composed of two components[19] and several authors have also distinguished different types of F hemoglobin[20, 21] by the use of an alkali denaturation method. We are in no position either to confirm or to refute such claims, since the techniques used in this study were different from those employed by the other investigators. In our experience the process of alkali denaturation becomes difficult to control when reagents of a pH lower than 12.7 are used. However, the close agreement of the results by the alkali denaturation technique used in this laboratory[4] and the electrophoretic measurements of the non-S hemoglobin fractions in the sickle-cell anemia hemolysates indicate to us that the alkali resistant compound probably represents the same component.

SUMMARY

A comparative study, by means of electrophoresis and alkali denaturation, of the non-S hemoglobin fraction of typical sickle-cell anemia hemolysates was performed. It could be shown that this fraction is almost entirely composed of the alkali resistant fetal (F) hemoglobin. Whether small amounts of nor-

mal adult (A) hemoglobin also exist in this fraction cannot be definitely decided due to the relative insensitivity of the methods employed. For practical purposes, the quantity of S hemoglobin in typical sickle-cell anemia hemolysates may be expressed as the difference between the total hemoglobin and the percentage of the F pigment. Two atypical cases of sickle-cell disease whose bloods contain relatively large amounts of A and F pigments, besides S hemoglobin, are presented.

The authors wish to acknowledge the technical assistance of Mr. James Eichelberger, B.S., who rendered technical assistance.

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STUDIES ON THE NORMAL SERUM PANAGGLUTININ ACTIVE AGAINST TRYPSINATED HUMAN ERYTHROCYTES

II. Relationship to Cold Agglutination

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IT HAS been demonstrated previously that normal human serum contains an auto-agglutinin active against trypsinated erythrocytes.[1, 2] This normal agglutinin has been found in all sera tested and has been a source of "false positive" reactions when trypsinated erythrocytes have been used as test cells for the detection of "incomplete" antibodies. The normal agglutinin differs from the abnormal varieties in that incubation of the test cells with serum at room temperature or at 37° C. causes dispersion of the cells and reversal of the agglutination, usually within an hour.[1, 2] This reversal phenomenon is not seen in the case of abnormal hemagglutinins; thus, sufficient incubation can eliminate false positive reactions and improve the utility of the trypsin technique in the study of "incomplete" antibodies.[3]

The nature of the agglutinin which acts against trypsinated erythrocytes is not known. It appears unlikely that "pseudo-agglutination" is involved, as the agglutinin can be absorbed out of serum by repeated treatment with trypsinated cells, and can then be eluted from the cells in pure form.[2] Other likely alternatives would be that it represents a nonspecific activity of some normal serum component; that it results from activity of another antibody to a related antigen; or that it is, as suggested by Morton and Pickles,[4] the thermal extension of cold agglutinin activity. The purpose of the present investigation was to study the relationship between this normal auto-agglutinin (NAA) active only against trypsinated erythrocytes and normal cold agglutinins of the "complete" and "incomplete" varieties.

METHODS AND MATERIALS

Blood was obtained from members of the hospital staff and from patients free of any evident hematologic disorder. No blood specimens were refrigerated unless specifically stated. The technique of blood collection, trypsinization, and reading agglutination was performed, as previously described,[2] save that Tryptar®* was substituted for purified, crystalline trypsin. The Tryptar® was measured in milligrams of activity rather than by weight.

Cold agglutinin titers were run against untreated and against trypsinated cells from the respective sera studied, i.e., the "auto" technique was used exclusively. Cell suspensions were incubated in the refrigerator at 4° C. for 16 hours, and the titer was taken as the highest dilution of serum giving a 1+ agglutination.

Coombs' tests were performed according to the method of Evans and Duane.[5] Two sets of tests were performed: the first on the cells just following collection; the second on cells subjected to refrigeration with their native serum. In the latter test the cells

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Received for publication, March 3, 1953.
*Kindly supplied by the Armour Laboratories, Chicago, Ill.

were washed three times with saline, and then a volume of cells was added to an equal volume of serum from which the cells were taken, and the mixture incubated at 4° C. for sixteen hours. The cells were then removed from the serum, washed three times with saline at room temperature, and tested against Coombs' serum as before. All Coombs' tests were run at room temperature.

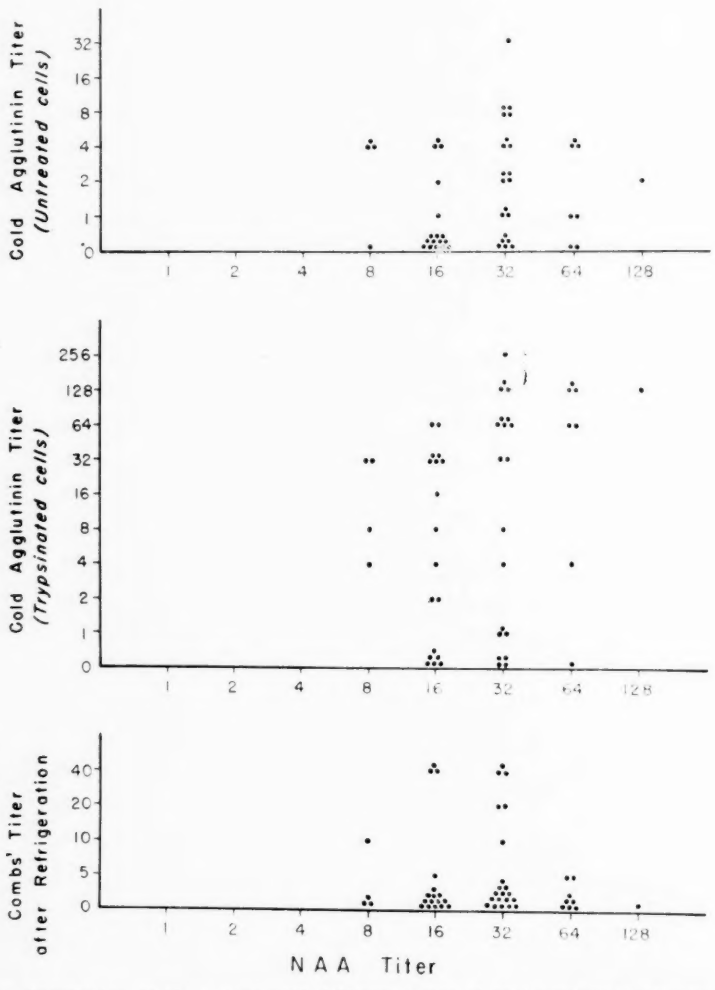
Cold Agglutinin Titer (Untreated cells)
Cold Agglutinin Titer (Trypsinated cells)
Coombs' Titer after Refrigeration
NAA Titer

Fig. 1.—Correlation of NAA titer with different types of cold agglutinin activity in fifty normal human sera.

RESULTS

Correlation Between Cold Agglutinin and Room Temperature Titers.—Fig. 1 shows the results of the various studies performed on fifty normal bloods. The NAA titer is plotted against the titer of cold agglutinin with untreated cells, with trypsinated cells, and against the Coombs' titer of cells refrigerated with their own serum. It can be seen that there is no correlation between these titers. It is also to be noted that incubation of cells in their own serum at re-

frigerator temperature is capable of producing a positive Coombs' test in about one-fourth of the cases, in agreement with the findings of Dacie[6] who suggested the presence of an "incomplete" cold agglutinin in certain normal sera. The sera which gave this reaction showed no difference in NAA titer.

Activity of Eluted Panagglutinin.—Five milliliters of serum was repeatedly absorbed at 20° C. with 0.3 ml. amounts of packed, trypsinated cells. Each cell aliquot was separated from the serum by centrifugation immediately after mixing, and promptly washed with saline three times to prevent spontaneous agglutination reversal. The resulting agglutinated cells were pooled, washed five times with normal saline, and suspended in 2 ml. of saline. To this suspension were added three drops of neutralized 0.1 per cent sodium cyanide solution, following which the agglutinated cells rapidly dispersed. It has been shown that sodium cyanide causes elution of NAA adsorbed onto trypsinated erythrocytes.[2] The suspension was then centrifuged, and the supernatant fluid removed and dialyzed for eighteen hours against five 200 ml. changes of normal saline. The resulting preparation contained an eluate of NAA. This eluate was then tested against trypsinated cells, and titers were obtained at room temperature just after mixing, and after sixteen hours of incubation at 4° C. As can be seen in Table I, the two titers were identical, indicating no increased activity of the eluate in the cold.

TABLE I. ACTIVITY OF NAA ELUATE AGAINST TRYPSINATED ERYTHROCYTES AT ROOM TEMPERATURE AND FOLLOWING REFRIGERATION

	1:1	1:10	1:20	1:40
20° C. immediate	4+	2+	1+	0
4° C. for sixteen hours	4+	2+	1+	0

Properties of Serum Absorbed with Trypsinated Cells at Room Temperature.—Five-tenths milliliter of serum was repeatedly exposed to 0.1 ml. amounts of packed, trypsinated cells. The cells were removed by centrifugation immediately following mixing. Fresh cells were used for each absorption, and the procedure was continued until the serum was free of NAA activity. Usually three to five absorptions were necessary. Two control samples of sera were also used: one was treated in an identical manner with untreated cells; the other was left unmodified. All three samples of sera were then tested for both NAA and cold agglutinin activity. Table II shows the results of these studies. It can be seen that treatment of the serum with both plain and trypsinated cells resulted in an insignificant loss of cold agglutinin activity which was probably due to an unavoidable dilution of the serum. In contrast, only the

TABLE II. EFFECT OF ABSORPTION OF NORMAL HUMAN SERA WITH TRYPSINATED CELLS AT ROOM TEMPERATURE

	NAA TITER		TITER AT 4° C.	
SERUM	UNTREATED	ABSORBED	UNTREATED	ABSORBED
1	1:16	0	1:32	1:8
2	1:32	1:2	1:64	1:16
3	1:16	0	1:16	1:8
4	1:32	0	1:64	1:32

trypsinated cells were able to absorb out NAA. It thus appears that NAA can be independently absorbed from the serum while leaving the cold agglutinin intact.

Activity of Serum Protein Fractions.—Serum was fractionated by a modification of the alcohol precipitation technique described by Lever and associates,[7] but the process was carried out in a refrigerated centrifuge, and the precipitates separated by centrifugation instead of filtration. The final products of fractionation were in each case dissolved in normal saline so that the volume was equal to that of the original serum. The solutions of protein fractions were dialyzed for eighteen hours against five 200 ml. changes of normal saline before use. Each fraction was tested for both NAA and cold agglutinin activity as described above.

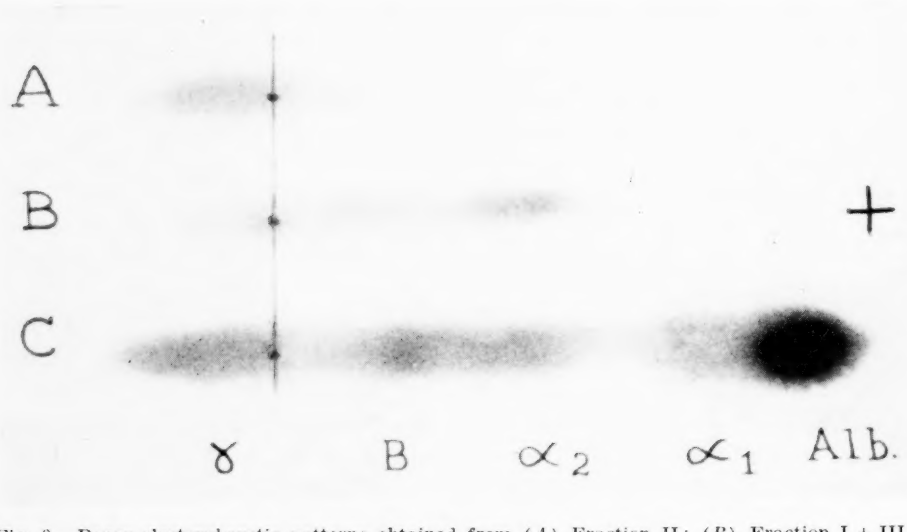

Fig. 2.—Paper electrophoretic patterns obtained from (*A*) Fraction II; (*B*) Fraction I + III; (*C*) normal human serum.

Fractions IV to VI showed no activity of either type. Fraction I + III showed NAA activity, and agglutination was noted after refrigeration. Inasmuch as there was no agglutination reversal due to separation of the reverser substance from the preparation,[2] it could not be determined whether this cold agglutinin activity represented persistence of the panagglutination or whether there was independent cold agglutinin activity in the fraction. Fraction II had no NAA activity, but was quite active after refrigeration. This finding is in agreement with the studies of Stats and Wasserman,[8] who identified the gamma globulin fraction as containing at least the abnormal type of cold agglutinin. Fig. 2 shows electrophoretic patterns on filter paper obtained from the active fractions. The patterns were obtained by the method of Kunkel and Tiselius.[9]

The serum from a patient with hemolytic anemia due to cold agglutinins was subjected to electrophoretic separation on paper. Simultaneous duplicate

runs were made, and one sample stained in the usual manner. The other sample was cut into sections corresponding to the various fractions seen on the stained specimen. Each section was eluted into 0.2 ml. of normal saline, and the respective eluates were tested for cold agglutinin against the patient's trypsinated erythrocytes. Only the gamma globulin fraction was active. An eluate of cold agglutinin was prepared from this same serum by absorbing it at 4° C. with untreated erythrocytes, washing the agglutinated cells with cold saline, and then eluting off the agglutinin into normal saline at 37° C. This eluate when subjected to paper electrophoresis gave a homogeneous spot at the gamma globulin position. Again, these results are in conformity with the previous investigations of Stats and Wasserman[8] that cold agglutinin is a gamma globulin. Attempts were made to perform similar studies with NAA. These efforts were unsuccessful, as the eluates contained insufficient protein for detection by the present techniques. Neither could sufficient agglutinin be isolated in any particular fraction to cause agglutination of trypsinated erythrocytes.

DISCUSSION

The data reported herein appear to distinguish clearly between the normal auto-agglutinin active against trypsinated erythrocytes and extended thermal activity of cold agglutinin. Several points of differentiation are evident. (a) There is no correlation between NAA and cold agglutinin activity, whether the latter be tested against untreated cells, trypsinated cells, or by its ability to render cells Coombs' test positive. (b) The activity of eluted NAA is not affected by cold. (c) Absorption of serum with trypsinated cells at 20° C. removes NAA activity, but does not significantly affect cold agglutination. (d) Cold agglutinin is found in the gamma globulin fraction of the serum proteins exclusively, whereas the panagglutinin appears in fraction I + III, which represents mainly alpha and beta globulins.

The nature of the NAA remains unclear. Although it has certain properties in common with antibodies—its ability to cause agglutination of cells, its property of being absorbed from serum with its appropriate substrate—it is not certain whether it is an antibody in the sense that it arises in response to antigenic stimulation. It is entirely possible that it is a normal serum component having as an independent side reaction the nonspecific properties which cause it to mimic the action of an antibody. If the panagglutinin is an antibody, the question arises as to the nature of the antigen which stimulates its appearance. One can speculate that the erythrocytes could become antigenic under circumstances in which their properties were temporarily modified. Proteolytic enzymes similar to trypsin have been demonstrated in human plasma,[10] and numerous bacteria and viruses have been found to affect the red cell in such a way as to alter their antigenic properties.[11, 12] This type of red blood cell alteration has been reported in vivo during the course of a urinary infection with streptococci. The patient's erythrocytes became temporarily panagglutinable by normal, compatible serum.[13] On the other hand, if panagglutination is a curious nonspecific protein reaction, the question arises as to its physiologic significance. Is it merely an interesting laboratory phenomenon with no significance in the under-

standing of disease, or can comparable modifications of serum proteins assume pathogenic importance? The development of so-called antibodies in acquired hemolytic anemia cannot be traced to known antigens; but they have been assumed to be antibodies on the basis of their immunological behavior.[14] To date it has not been possible to produce auto-antibodies associated with acquired hemolytic anemia in the experimental animal, although some investigators have succeeded in rendering the erythrocytes of animals Coombs' positive by injection of autologous cells.[15, 16]

An interesting and practical by-product of the present study was the finding that a positive Coombs' test can be produced in a significant number of normal individuals by refrigeration of their cells in their own serum. Confirming the findings of Dacie,[6] the cells did not become Coombs' negative even after warming to incubator temperature. It is thus evident that the interpretation of a positive Coombs' test is valid only on specimens of blood which have not been refrigerated prior to the separation of cells from serum. Studies reporting a positive Coombs' test in conditions where auto-agglutinins are not anticipated must be evaluated in light of these findings.

SUMMARY

The normal serum panagglutinin active against trypsinated red blood cells was compared to cold agglutinin, and its activity was found to differ in the following ways: (a) There was no correlation between cold agglutinin titer and panagglutinin titer. (b) The activity of panagglutinin was not affected by cold. (c) Panagglutinin could be absorbed from serum at room temperature without significant loss of cold agglutinin activity. (d) Fractionation of serum showed pannagglutinin activity only in fractions I + III, whereas cold agglutinin was found in fraction II.

The significance of these findings is discussed.

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CONGENITAL FAMILIAL DEFICIENCY OF THE STABLE PROTHROMBIN CONVERSION FACTOR; RESTUDY OF CASE ORIGINALLY REPORTED AS "IDIOPATHIC HYPOPROTHROMBINEMIA"

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IT HAS become evident with Owren's exhaustive study of Factor V[1] that the conversion of prothrombin to thrombin does not occur merely with the aid of thromboplastin and calcium. More recently several investigators[2-5] demonstrated the existence of another factor influencing the conversion of prothrombin. Because of the confusing terminology applied to these two clotting elements we will adopt the simplified nomenclature suggested by Owen and associates[6] where the term "labile factor" (L.F.) is used for Owren's factor V, and the term "stable factor" (S.F.) for the more recently discovered accelerator. The adjectives labile and stable define resistance to storage. Most investigators[7] agree that labile factor (Quick[8]), Factor V, or proaccelerin (Owren[5]), plasma accelerator globulin (Seegers[9]), and prothrombin accelerator (Fantl[10]) are one single entity. Stable prothrombin conversion factor is a synonym for serum prothrombin conversion accelerator (SPCA, Alexander[2]), Factor VII (Koller[3]), co-thromboplastin (Mann[4]) and convertin (Owren[5]).

The present study concerns the case reported from this institution in 1949 as "idiopathic hypoprothrombinemia."[11] With the aid of modern laboratory tests, and in the light of present-day knowledge of blood coagulation, it will be shown that the abnormality in this patient actually is a deficiency of the stable prothrombin conversion factor rather than hypoprothrombinemia. For the clinical data we refer to the previous publication. Since then the patient has continued to have intermittent subcutaneous and intramuscular hemorrhages. An interesting feature has been the occurrence of low abdominal pains followed by the syndrome of a paralytic ileus of three to six days' duration at monthly intervals; these episodes are probably related to ovulation which is accompanied by some intra-abdominal extravasation of blood. The patient, who is now 24 years of age, has not developed any crippling hemarthrosis.

METHODS

Collection and Processing of Blood, Plasma, and Serum.—Silicone-coated syringes, needles, test tubes, and pipettes were used for all experiments except for the determination of the venous clotting time and the prothrombin consumption test which were performed in plain glass tubes. In order to avoid contamination with tissue juice the blood was collected by a two-syringe technique, and the content of the first syringe was discarded. For the preparation of plasma the second syringe contained either 0.1M sodium oxalate or 3.8 per cent sodium citrate solution, depending on the test to be performed. All blood samples were mixed with 1/10 volume of anticoagulant.

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Received for publication, Mar. 30, 1953.

$BaSO_4$ Plasma was prepared by adding 700 mg. of $BaSO_4$* to 10 ml. of oxalated plasma. This mixture was gently shaken for a period of five minutes and the $BaSO_4$ centrifuged out at 2,000 r.p.m. for ten minutes.

$BaSO_4$ Serum was prepared by the same technique. For complete adsorption of prothrombin and S.F., it was necessary to mix the serum with 1/10 volume of 0.1M sodium oxalate before adding $BaSO_4$.

Forty-eight Hour Old Normal Serum was used as a source of S.F. This serum was free of thrombin and contained only traces of prothrombin and L.F. Normal blood was collected under sterile conditions and left at 37° C. for forty-eight hours in a plain glass tube. The clot was then rimmed and the serum separated by centrifugation.

L.F. Deficient Plasma was obtained by storing normal oxalated plasma at 20 to 24° C. for eleven days under sterile conditions. If not stated otherwise, all other plasma samples were tested within six hours after venipuncture.

Modified Two-Stage Prothrombin Determination.—For the determination of the rate of conversion and the concentration of prothrombin the two-stage method of Ware and Seegers[12] was applied with the following two modifications: first, the plasma to be tested was not defibrinated with bovine thrombin because, as will be shown later, the commercial thrombin preparation† contained S.F. as a contaminant which caused a definite acceleration of the conversion of prothrombin. The appearance of fibrin in the incubation mixture after addition of the diluted plasma had no disturbing effect because it always occurred after the shortest time had been reached, i.e., after all the prothrombin had been converted to thrombin. This was true, however, only if the final plasma dilution was set at 1:300; all through our experiments the plasma was studied in this concentration. Second, the addition of $BaCO_3$ treated bovine serum as a source of active Ac-globulin was omitted in order to obtain a curve which represented solely the mechanism of coagulation of the patient's native plasma. This variation of Ware and Seeger's prothrombin determination will be hereafter called "modified two-stage method."

Difco Rabbit Brain Thromboplastin was used throughout.

Armour's Bovine Fraction I served as fibrinogen substrate. One hundred milligrams of dry powder was dissolved in 9.5 ml. of 0.9 per cent NaCl solution and 0.5 ml. of 1.72 per cent imidazole buffer.[12]

Commercial Bovine Thrombin‡ was used in a concentration of 25 units per milliliter of normal saline.

Purified Bovine Thrombin was obtained by adsorbing 500 units of commercial thrombin dissolved in 9.0 ml. of 0.9 per cent NaCl solution and 1.0 ml. 0.1M sodium oxalate solution with 500 mg. of $BaSO_4$. The mixture was gently shaken for five minutes and the $BaSO_4$ removed by centrifugation. This purification procedure resulted in a loss of approximately half the thrombin activity, but the final concentration of the purified product could be set at 25 units per milliliter by starting with a double strength solution.

Prothrombin Consumption Test.—Blood was collected in a siliconized syringe and distributed into a series of 75 by 8 mm. dry glass tubes calibrated to 2.0 ml. All tubes were placed in a water bath at 37° C. At fifteen-minute intervals 0.2 ml. of 3.8 per cent sodium citrate solution was added to successive tubes and the citrated serum was obtained by centrifugation (2,000 r.p.m. for five minutes). The concentration of prothrombin was determined on each sample of the patient's serum (0.1 ml.) with the modified two-stage method after addition of forty-eight hour old normal serum as a source of S.F.

Definitions.—The use of the terms *conversion* and *consumption* of prothrombin deserves clarification. Conversion is used to indicate the changes in prothrombin concentration against time as measured by the modified two-stage determination of prothrombin in plasma. In this test three clotting factors (thromboplastin, calcium, and fibrinogen) are kept constant by adding the appropriate reagents. Consumption of prothrombin is used to describe the decreases in prothrombin concentration occurring during spontaneous coagulation in glass tubes at 37° C.

*Merck and Company, Inc., Rahway, N. J.
†Parke, Davis & Company, Detroit, Mich.
‡Parke, Davis & Company, Detroit, Mich.

LABORATORY STUDIES

Results of Two-Stage Prothrombin Determinations.—In analyzing two-stage data one should remember that the slopes of the curves obtained with this method indicate the rate of prothrombin conversion. In turn, the rate of conversion depends upon the concentration of one or more accessory factors (e.g., L.F. and S.F.). The shortest value obtained on the ordinate is the index of prothrombin concentration.

The patient's curve indicates a very slow activation of prothrombin to thrombin. However, the concentration of prothrombin was 240 units per milliliter which is within normal limits for our laboratory. This value was obtained fifteen minutes after mixing the patient's diluted plasma with the incubation mixture, as opposed to three or four minutes necessary for normal plasma.

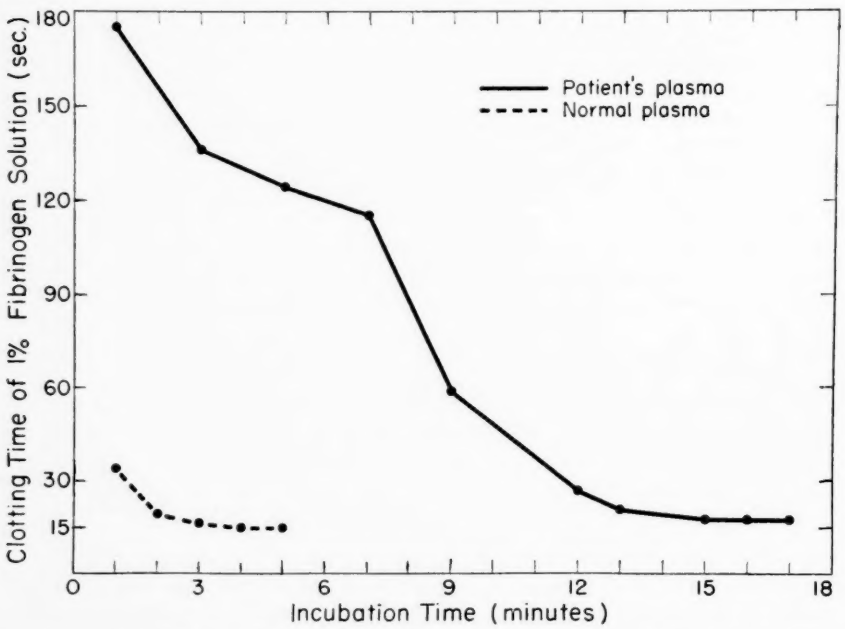

Fig. 1.—Prothrombin determination on citrated plasma with the modified two-stage method.

Neither normal $BaSO_4$ treated plasma nor normal $BaSO_4$ treated serum had any effect on the retarded prothrombin conversion rate of the patient's plasma. However, untreated normal serum stored for forty-eight hours at 37° C. had a rectifying effect. This serum contained less than 1 unit per milliliter of prothrombin. The concentration of prothrombin in the patient's plasma determined after addition of normal serum was 306 units per milliliter, a value which was slightly higher than when the patient's plasma was tested alone. The lower value obtained without added serum was probably due to antithrombin activity which is known to be more marked when the conversion

of prothrombin is slow. Serum aged for nine days was practically as effective as forty-eight hour old serum. The data in Fig. 2 indicate that the patient's plasma is deficient in a factor which is present in normal serum. This factor is stable to storage and is adsorbed by $BaSO_4$; in other words, it has the characteristics of the clotting entity referred to above as stable factor (S.F.).

Indirect evidence for a deficiency of S.F. was also found by comparing the effects of the patient's serum and of normal serum (both aged for forty-eight hours) on the patient's own two-stage prothrombin conversion rate (Fig. 3).

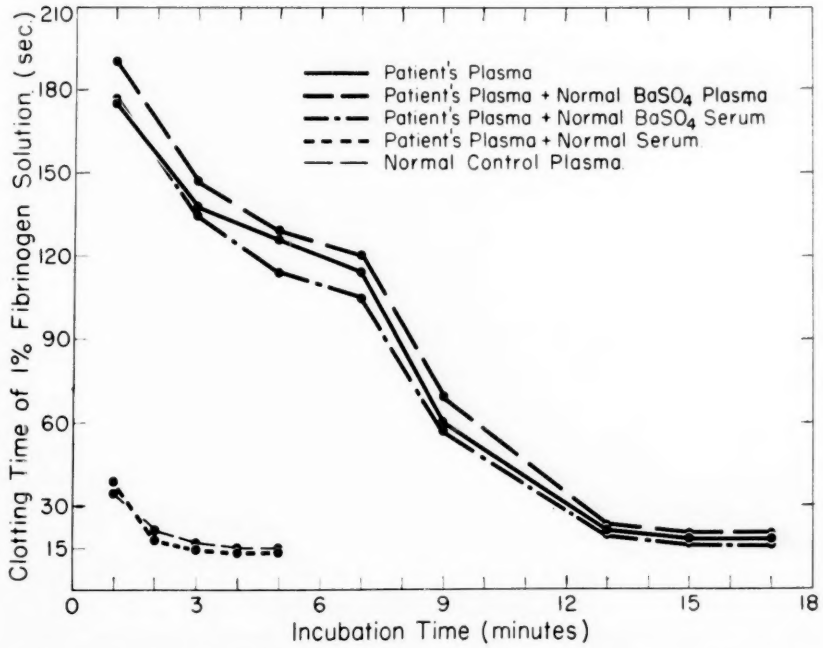

Fig. 2.—Comparison of the effect of normal serum, $BaSO_4$ serum, and $BaSO_4$ plasma on the patient's prothrombin conversion rate. Technique: modified two-stage method. 0.1 ml. of normal serum, $BaSO_4$ serum, or $BaSO_4$ plasma added to 0.1 ml. of patient's plasma immediately before the two-stage determination. Dilution of patient's plasma set at 1:300 in all tests.

The effect of the patient's serum on the activation of prothrombin in its mother plasma was minimal. This indicates that the serum's content of S.F. was very low.

It has not been possible so far to study the effect of the S.F. or of a possible precursor of it in plasma, because the necessity of removing prothrombin from plasma (with $BaSO_4$ or another adsorbent), before testing for S.F. activity, has always been accompanied by the removal of the S.F. as well. A separation of the two plasma components by Seitz filtration described by Owren[13] was not attempted. Normal serum aged for forty-eight hours was found to be an adequate source of S.F. At the same time, this serum contained only traces of

prothrombin, and L.F. and was free of thrombin. The S.F. was inactivated by heating at 56° C. for forty-five minutes or at 60° C. for ten minutes. It was not dialyzable.

Exclusion of L.F. Deficiency.—Fresh normal $BaSO_4$ plasma which contains L.F. but not S.F. or prothrombin failed to correct the defect of the patient's plasma. This excluded a deficiency of L.F. A further demonstration that the patient's plasma had a normal concentration of L.F. is illustrated in Fig. 4. Both normal $BaSO_4$ plasma and the patient's $BaSO_4$ plasma had essentially the same restorative effect on the retarded and, in this instance, incomplete prothrombin conversion of stored normal plasma deficient in L.F.

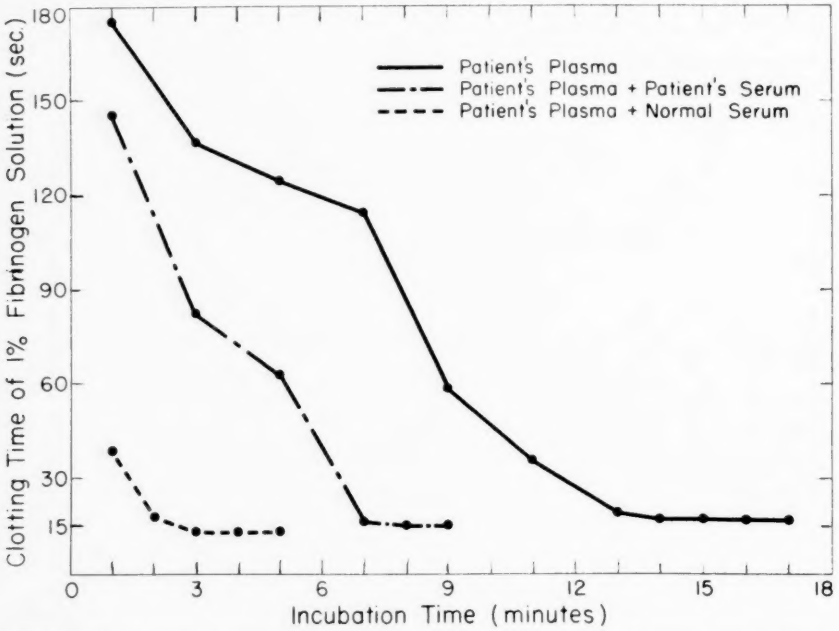

Fig. 3.—Effect of normal serum vs. patient's serum on the patient's prothrombin conversion. Technique: As outlined under Fig. 2.

Patient's Prothrombin Consumption During Spontaneous Coagulation.—The concentration of prothrombin in the patient's serum was determined periodically every fifteen minutes after venipuncture over a period of two hours. The prothrombin consumption was within normal range. Theoretically, in view of the curve in Fig. 1, one would have expected a slow consumption. An attempt to explain this paradoxical situation was made by Alexander,[14] who felt that the influence of what he calls SPCA (which is identical with S.F.) is limited to the very early phase of clotting, a range of time which cannot be analyzed with the test the results of which are given in Fig. 5. In this regard blood deficient in S.F. behaves quite differently from hemophilic blood in which the consumption of prothrombin is usually less than 10 per cent during the first two hours. It is also known that L.F. deficiency delays prothrombin consumption.[15]

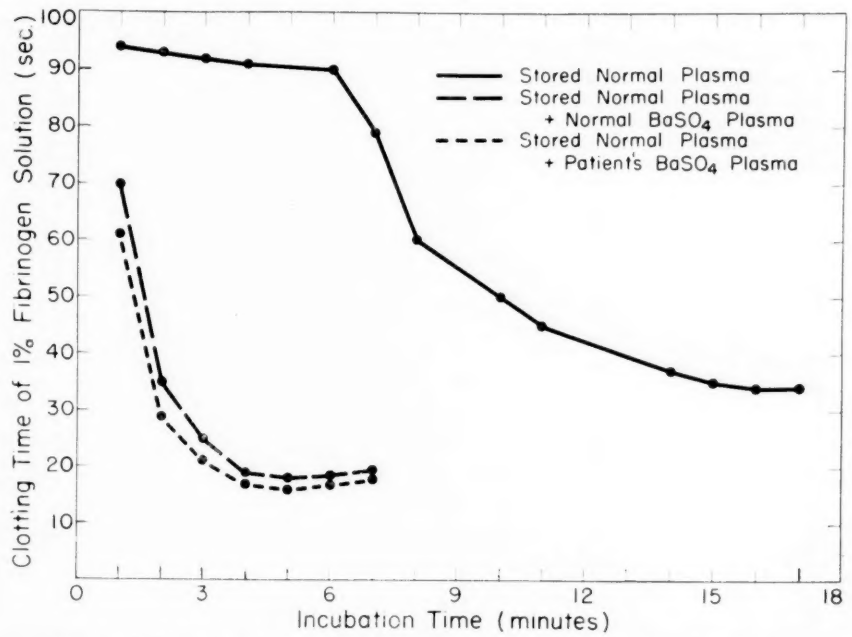

Fig. 4.—Effect of patient's $BaSO_4$ plasma vs. normal $BaSO_4$ plasma on prothrombin conversion of L.F. deficient plasma. Technique: Modified two-stage method. 0.1 ml. of normal stored plasma mixed with 0.1 ml. of patient's or normal $BaSO_4$ plasma immediately before two-stage determination. Final dilution of stored plasma: 1:300.

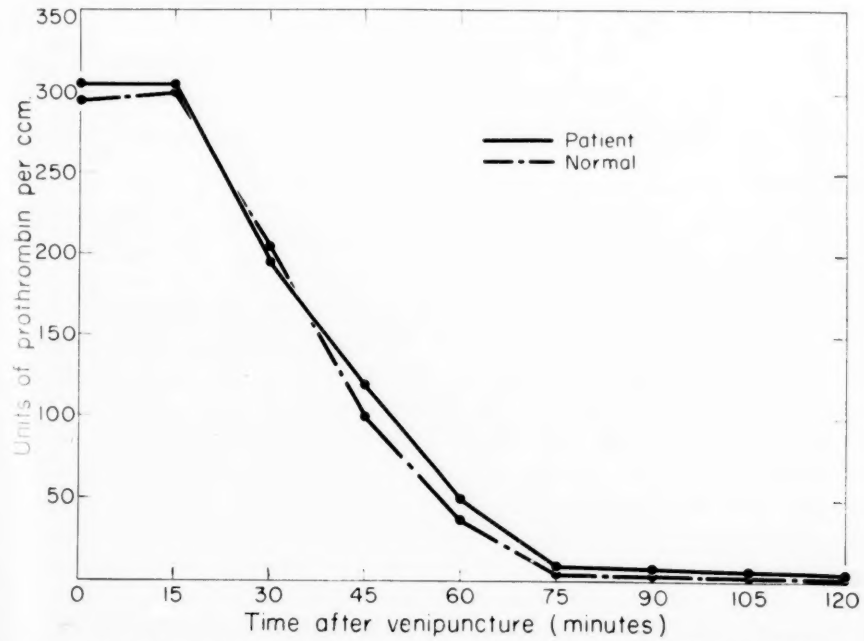

Fig. 5.—Patient's prothrombin consumption during spontaneous coagulation.

Effect of Defibrination with Crude and Purified Commercial Thrombin on the Patient's Prothrombin Conversion Rate.—Very early in the course of this study a striking discrepancy was observed between the prothrombin conversion rate of the patient's plain plasma and of this plasma defibrinated with commercial thrombin. Because of the theoretical and practical implications of these, at first puzzling, results, a separate study was undertaken in an attempt to explain this phenomenon.

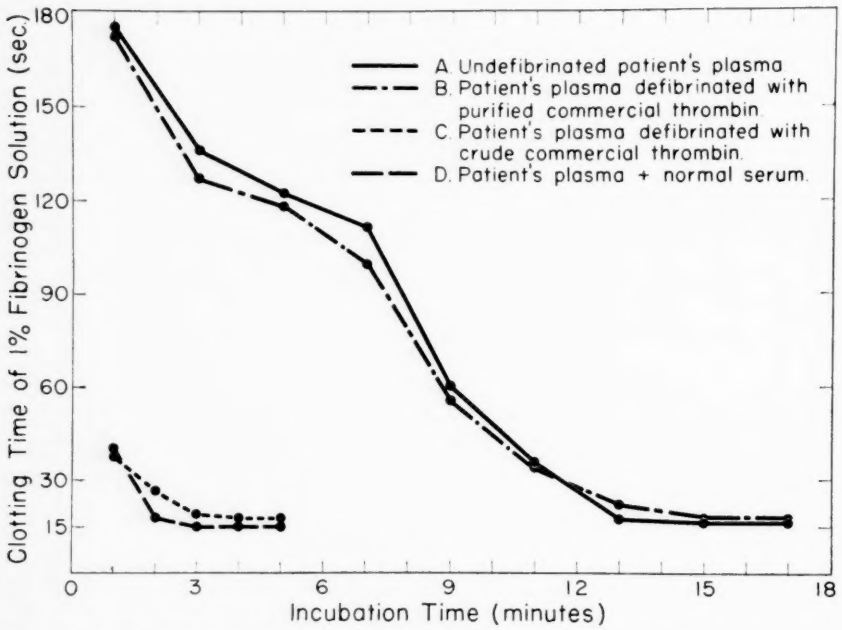

Fig. 6.—Effect of defibrination with thrombin on the patient's prothrombin conversion rate.

The use of commercial thrombin to defibrinate the patient's plasma before running a two-stage determination of prothrombin resulted in a curve (curve *C*, Fig. 6) which was quite different from the one obtained with undefibrinated plasma (curve *A*, Fig. 6). The rate of prothrombin conversion was very rapid and practically identical with the rate obtained after adding normal forty-eight hour old serum as a source of S.F. to the patient's plasma (curve *D*, Fig. 6). It was therefore suspected that the commercial thrombin preparation contained enough S.F. to account for this result. An attempt was then made to eliminate the S.F. from the thrombin solution by adsorbtion on $BaSO_4$. After repeated trials with various concentrations of thrombin and $BaSO_4$ and changes in incubation times, it was finally possible to obtain an active thrombin solution which was free of S.F. by the method outlined initially. Curve *B* (Fig. 6) obtained with the patient's plasma defibrinated with $BaSO_4$ treated thrombin was practically identical with the curve originally obtained with undefibrinated plasma (curve *A*, Fig. 6). These data indicate the presence of S.F. as a contaminant of commercial bovine thrombin. This fact should be

TABLE I. ROUTINE TESTS OF HEMOSTASIS

	PATIENT	NORMAL VALUES FOR THIS LABORATORY
Bleeding time (Ivy)	14 minutes, 00 seconds	1 to 6 minutes
Clotting time (Lee-White)	31 minutes, 00 seconds	12 to 20 minutes
Prothrombin time (Quick)	67.5 seconds	15 to 16 seconds
Recalcification time	690 seconds	75 to 150 seconds
Platelets (Reese-Ecker)	220,000/cu. mm.	
Clot retraction	Normal	
Fibrinogen	340 mg. per cent	
Capillary fragility (Rumpel-Leede) negative.		

kept in mind in order to avoid false conclusions regarding the possible influence of commercial thrombin on prothrombin conversion. Thus, in our case one could readily have drawn the faulty conclusion that thrombin had the same effect as normal serum on the patient's prothrombin conversion rate. Actually this effect was not caused by the thrombin per se, but by the S.F. present as a contaminant. It should be mentioned, however, that commercial thrombin can safely be used for determination of the *concentration* of prothrombin by the two-stage method, because its content of S.F. does not affect the shortest time obtained with this method. If one desires information regarding the *rate of conversion* of prothrombin one should use purified thrombin or test undefibrinated plasma.

Results Obtained with the One-Stage Method for Prothrombin Determination.—For the purpose of comparison of the data obtained in our case with other

TABLE II. PROTHROMBIN TIMES OF PATIENT'S PLASMA OR SERUM AFTER ADDITION OF VARIOUSLY TREATED NORMAL PLASMA AND SERUM

PARTS OF PLASMA OR SERUM						
PATIENT'S PLASMA	NORMAL PLASMA	NORMAL SERUM 48 HOURS OLD	PATIENT'S SERUM 48 HOURS OLD	NORMAL $BaSO_4$ PLASMA	NORMAL $BaSO_4$ SERUM	PROTHROMBIN TIME* IN SECONDS
1						65.5
	1					15.0
1		1				14.0
1			1			52.5
1				1		75.1
1					1	73.3

*The one-stage prothrombin time of plasma collected in siliconized equipment with the technique outlined earlier in this paper is slightly longer than with plasma collected with dry uncoated syringes. The glass surface probably initiates "invisible coagulation" with activation of conversion factors.

TABLE III. PROTHROMBIN CONSUMPTION

TIME AFTER VENIPUNCTURE IN MINUTES	SERUM PROTHROMBIN TIME IN SECONDS
0 (plasma prothrombin time)	14.0
15	14.8
30	16.6
45	17.3
60	24.0
90	26.9
120	30.1

Technique: One-stage prothrombin time on 0.1 ml. of patient's serum after addition of 0.1 ml. of normal forty-eight hour old serum as a source of S.F. If the S.F. was omitted the values were very erratic.

publications on clotting defects in which the one-stage method has been used exclusively or at least partially, our results with this method are summarized in Tables II and III.

All the results obtained with the one-stage method confirmed the data obtained with the two-stage method. The advantage of the one-stage method lies mainly in the rapidity with which the test can be done, while the more cumbersome two-stage method allows a much better and more precise evaluation of the dynamics of coagulation. It is well known that the times obtained with the one-stage method depend on the concentration of prothrombin, L.F., S.F. and fibrinogen (calcium and thromboplastin being kept constant). If the fibrinogen is within normal range, the times obtained are a combined index of

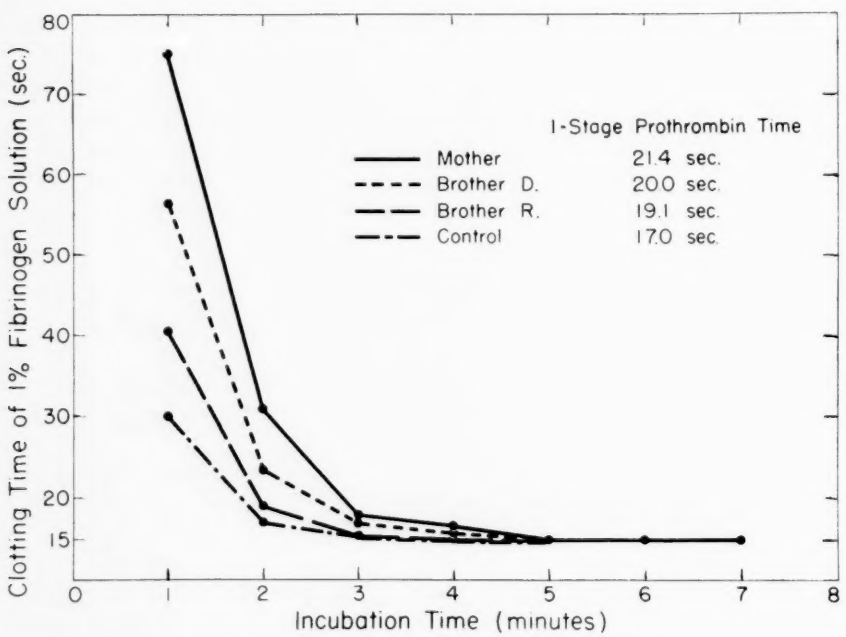

Fig. 7.—Study of other family members.

concentration and speed of conversion of prothrombin. The most valuable information obtainable with the two-stage method is the differentiation between the rate of conversion and the concentration of prothrombin in the same test. This cannot be done with the one-stage method.

Study of Other Family Members.—In our previous publication the one-stage prothrombin time was found to be prolonged in the patient's mother and in three siblings. The patient's mother and two siblings were restudied with the modified two-stage method, and all of them showed a normal concentration of prothrombin with a moderate delay of prothrombin conversion (Fig. 7). This abnormality was corrected by addition of S.F. Hence this condition has

definitely a familial character, and the type of inheritance is probably dominant. Why our patient has a marked deficiency in S.F. and all other members of the family only a minimal clotting defect remains unexplained.

DISCUSSION

The careful and systematic study of congenital hemorrhagic disorders has added valuable information of our understanding of the mechanism of blood coagulation. The existence of two nonprothrombin factors influencing the conversion of prothrombin to thrombin has been substantiated by Owren's description of parahemophilia (L.F. deficiency) and Alexander's more recent report on congenital SPCA deficiency. While several cases of parahemophilia have been added to Owren's first case, there are only three detailed published reports of S.F. deficiency (Alexander,[14] Owren[16, 19]); most recently Stefanini[17] alluded to an additional case under study. There can be little doubt that the clotting defect described in our patient is identical with that in Alexander's and Owren's cases, thus supporting the assumption that SPCA, convertin, and S.F. are synonymous. The physical characteristics of these factors and their effect on the clotting mechanism are identical. Occasional conflicting data only are apparent and reflect differences in test methods. Alexander[14] reported a normal conversion rate of prothrombin using the orthodox two-stage method without Ac-globulin supplements; similar results were obtained in our patient when crude commercial thrombin was used to defibrinate the plasma. However, when the patient's "pure" plasma was tested, without preliminary defibrination, its rate of prothrombin conversion was markedly delayed. The addition of crude commercial thrombin corrected this delay because of the presence of S.F. as a contaminant. When the patient's plasma was tested after defibrination with purified thrombin, freed of S.F., the conversion of prothrombin was retarded. A possible source of S.F. contamination in Alexander's study was the crude lung thromboplastin which was used originally. In a later report the same investigator[18] used purified thromboplastin and found a moderately retarded prothrombin conversion. The rabbit brain thromboplastin used in our studies was found to contain only minimal traces of S.F. Various batches of the commercial Difco preparations varied somewhat in their content of S.F. It is obvious that any thromboplastin prepared from animal organs contains traces of blood, thus traces of S.F. Thromboplastins of pulmonary or placental origin contain more S.F. than products obtained from brain. In the latter the S.F. can be inactivated by heating at 60° C. for ten minutes. Due to the relative heat stability of brain thromboplastin, this procedure does not affect its activity.

The question whether L.F. or S.F. act as accelerators or whether their action is stoichiometric has not been settled thus far. In regard to S.F. the observations contained in the present report add valuable information. First, a deficiency in S.F. resulted in delayed conversion of prothrombin, but the final yield of thrombin was normal if sufficient time was allowed for complete conversion. In other words, prothrombin conversion was defective in respect to

time and not in respect to quantity. Second, the consistent effectiveness of S.F. contained in normal serum even several days after spontaneous coagulation suggested that S.F. was used but to a small extent, if at all, during clotting. These two facts strongly suggest a catalytic action of the S.F. Therefore, S.F. appears to be a true accelerator of prothrombin conversion.

From a clinical standpoint this report confirms Owren's first description[19] of the familial incidence of this congenital hemorrhagic diathesis. The necessity of differentiating idiopathic hypoprothrombinemia from deficiency of L.F. or S.F. among the congenital clotting defects with a prolonged one-stage prothrombin time has become imperative. It is probable that if the other cases thus far reported as "idiopathic hyproprothrombinemia" would be restudied with modern methods, a certain number of them would reveal a defect in prothrombin conversion caused by a deficiency of S.F. or L.F.

SUMMARY

A familial, congenital hemorrhagic diathesis due to deficiency of the stable prothrombin conversion factor (S.F.) has been presented. The abnormality is identical with the defect described by Alexander and associates as "congenital SPCA deficiency" and by Owren as "congenital hypoproconvertinemia."

There is good evidence that the action of the stable factor on prothrombin conversion is accelerating in type.

Commercial thrombin was found to contain enough S.F. as a contaminant to correct the delayed prothrombin conversion in this case.

The authors gratefully acknowledge the advice and assistance of Dr. C. J. Watson.

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THE TOXIC EFFECTS OF MODIFIED HUMAN GLOBIN

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MODIFIED human globin[1] is a product of the controlled alkaline hydrolysis of globin derived from human erythrocytes. Chemical studies[2] have indicated that this product is a mixture of several substances, of which 80 per cent consists of protein fragments with a molecular weight of about 34,000. Measurements of the plasma volume have shown significant increases after intravenous administration of solutions of this material to normovolemic human subjects.[3] Globin solutions have been given successfully in the treatment of hemorrhagic shock[4] and hypoproteinemia.[5, 6] The reported toxic reactions produced by this material have in general been minor. The chief reaction has been the appearance during the infusion of flushing and pruritus which tended to disappear when the rate of infusion was slowed. A few recipients of modified human globin have developed pyrogenic reactions.[4, 7, 8, 9, 10, 11] Two patients voided red urine after receiving modified human globin.[8, 10]

Since none of the generally available forms of protein for intravenous administration is entirely satisfactory, a study was undertaken to determine the advantages and disadvantages of modified human globin, both as a nutritional supplement for intravenous use, and as a plasma substitute.

METHODS

Solutions of modified human globin were given intravenously to four patients. (Modified human globin was supplied through the courtesy of Sharp and Dohme, Inc., Philadelphia, Pa.) Two patients received large rapid infusions of globin such as might be given if the material were being used to treat hemorrhagic shock. One of these patients, J. S., also received a rapid infusion of 50 Gm. of serum albumin in a control study. Two other patients were given slow daily infusions to simulate the treatment of hypoproteinemia. Data on the composition of the infusions and the rates of administration are given in Table I. The patients who received repeated infusions of globin were studied by the metabolic balance technique. In the balance studies, elementary analyses were performed on the diets and collections of urine and feces. Standard clinical pathologic and chemical methods were used. Urine protein was determined by the method of Peters and Van Slyke.[12] The analyses of blood and urine for globin were done by the method of Kendall and Abell described by Sample, Strumia, and Satta.[13] The filter paper electrophoresis patterns were obtained by the method of Durrum.[14] Conventional electrophoretic analyses were carried out in sodium barbital buffer of ionic strength 0.1 at pH 8.6.

RESULTS

Rapid infusions of two different lots of globin produced identical reactions in two patients (J. S. and R. LaP.). Shortly after the infusion was

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Received for publication, March 20, 1953.

started there appeared erythema and pruritus, soon followed by urticaria which became generalized. These reactions subsided partially when the rate of infusion was decreased. Although one patient seemed to be benefited by the administration of 4 mg. of chlorprophenpyridamine (Chlortrimeton), prophylactic administration in the second patient failed to prevent the reaction. Both patients had a slight chill about an hour after the infusion was completed. Body temperatures reached a peak of approximately 100° F. three hours after the infusions, but fell again to normal in the next three hours. Both patients experienced anorexia and mild nausea during the periods of infusion and of fever. The patients (W. P. and F. S.) who received slow daily infusions of globin both developed marked anorexia after the first few infusions. Patient F. S. experienced mild generalized pruritus with the later infusions. In this patient the infusions had to be discontinued after nine days because of the appearance of gross hematuria.

TABLE I. COMPOSITION AND METHODS OF ADMINISTRATION OF INFUSIONS OF MODIFIED HUMAN GLOBIN

PATIENT	COMPOSITION OF INFUSION	GLOBIN LOT NO.	INFUSION RATE (GM./MIN.)	NUMBER OF INFUSIONS	TOTAL AMOUNT INFUSED (GM.)
W. P. Chronic hepatitis	2% and 4% globin in 5% dextrose solution	95619	0.17 and 0.33	6	160
F. S. Amputation	4% globin in distilled water	95619	0.09	9	288
R. LaP. Rheumatoid arthritis	8% globin in distilled water	52-1	0.53	1	64
J. S. Chronic hepatitis	8% globin in distilled water	95619	0.53	1	64
J. S. Chronic hepatitis	25% albumin as supplied	--	0.42	1	50

The rapid infusions of globin produced an immediate rise in the hematocrit (Fig. 1). The serum globulin rose, while the serum albumin fell. All these changes returned to control values in t enty-four hours. In patient J. S. the plasma hemoglobin did not change after globin infusion. The slow repeated infusions of globin caused no change in the hematocrit or the serum albumin, but after several days of infusion there was a substantial rise in serum globulin which did not return to control values until ten days after the infusions were discontinued (Fig. 2). Serums containing increased globulin gave negative chemical tests for globin, even when tested only twelve hours after globin infusion. The electrophoretic analyses of serums from patient W. P. showed that the rise in globulin was due primarily to an increase in gamma globulin. Both patients W. P. and F. S. showed a marked rise in the erythrocyte sedimentation rate which paralleled the rise in serum globulin. This rise could not be duplicated in vitro by the addition of globin to blood. Patient J. S. showed a more positive cephalin flocculation test after globin infusion, while the thymol turbidity measurement fell, although the other patients showed no changes in the liver function tests.

All patients receiving globin showed proteinuria (Figs. 1 and 2). Protein excretion was marked during globin infusion, but decreased rapidly

afterward. No protein was present in the urine eighteen hours after the infusion. By chemical analysis approximately one-half of this protein was globin. Fig. 3 shows the filter paper electrophoresis patterns of urinary protein from patients W. P. and F. S. These patterns suggested that some serum proteins were excreted by the kidney in addition to the globin. Daily morning urine specimens were examined in patients W. P. and F. S. In both, red cells, white cells, and coarsely granular casts appeared in more than normal numbers

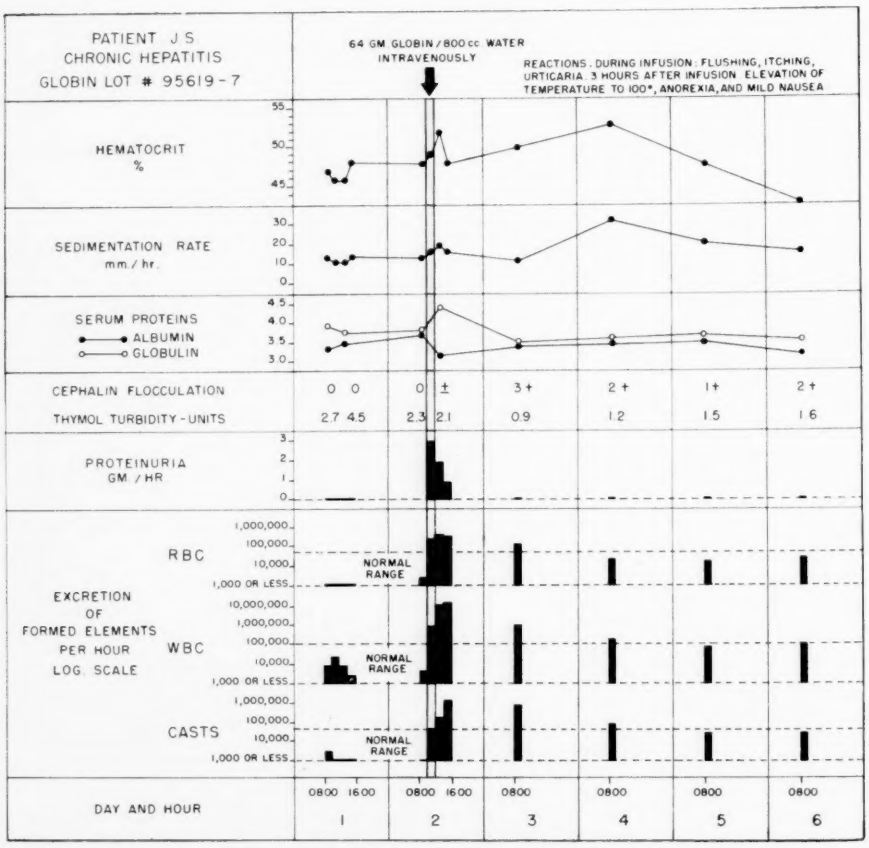

Fig. 1.—The effects of a single rapid infusion of modified human globin.

during the globin infusions and persisted for several days after globin was discontinued. Quantitative counts of the sediment from timed urine collections were performed during the studies on patients J. S. and R. LaP. There were immediate marked increases in all the formed elements with the infusion of globin. The numbers of formed elements excreted did not return to control values in four days. The control albumin infusion given to patient J. S. produced no changes in the excretion of formed elements. As described above, patient F. S. developed gross hematuria (red urine, loaded with red cells on microscopic examination, which showed a positive benzidine test) after nine

daily infusions of globin. Endogenous creatinine clearances showed no changes after a single rapid infusion of globin in patient R. LaP. In patient W. P., however, who received repeated globin infusions, the twenty-four hour clearance of endogenous creatinine fell approximately 30 per cent during the infusions of globin and had not risen again six days after the infusions were discontinued.

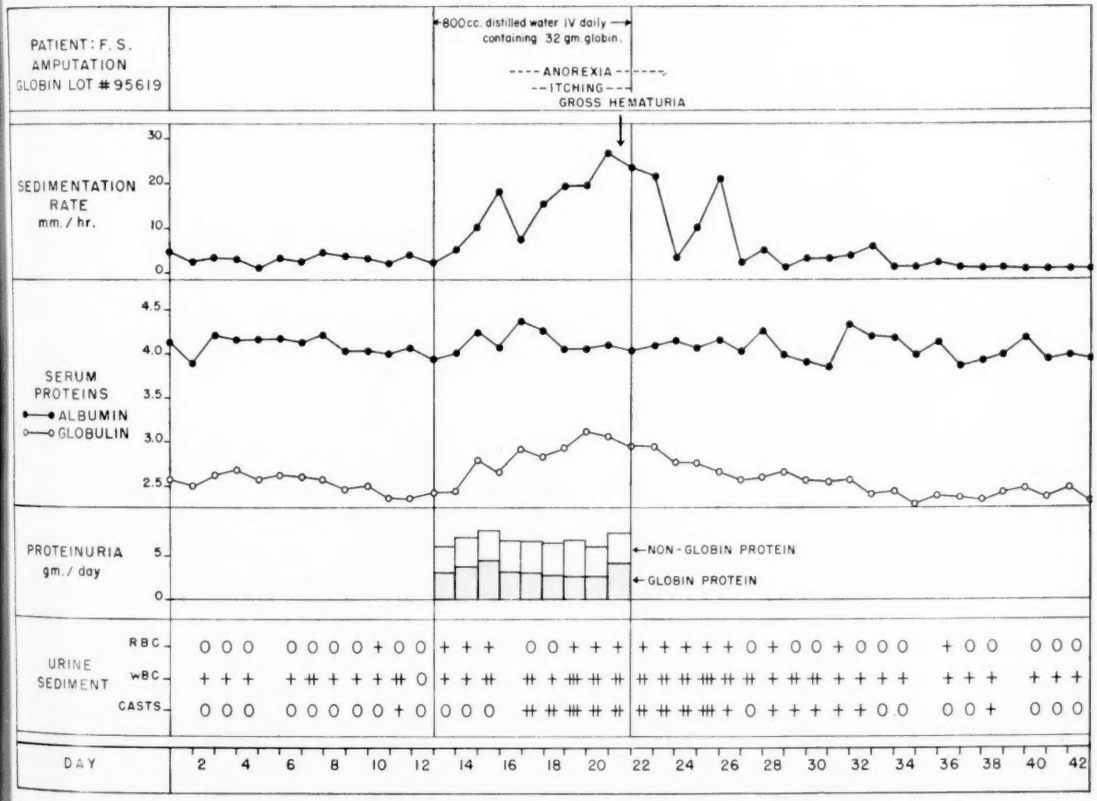

Fig. 2.—The effects of repeated daily infusions of modified human globin on the erythrocyte sedimentation rate, serum proteins, urine protein, and urine sediment. Grading of urine sediments: + rare, occasional; ++ 1-10 per hpf; +++ 10-50 per hpf, many; ++++ loaded.

Although balance studies were performed on two patients, the results in W. P. were impaired because the anorexia produced by the globin prevented the maintenance of a constant food intake. Patient F.S. retained most of the infused nitrogen during the first four days of globin administration (Fig. 4 and Table II). Thereafter the nitrogen excretion increased day by day until on the ninth day the subject was in negative nitogen balance. The negative balance persisted for about six days after the infusions were discontinued. Of the 41.5 Gm. of nitrogen given in the globin infusions, 4.7 Gm. was excreted as unchanged globin. During and after globin administration the patient excreted 25.3 Gm. of nonglobin nitrogen in excess of the average output before

globin was given. Thus 11.5 Gm., or 28 per cent of the globin nitrogen, was retained by the subject. Considerable potassium was retained while globin was administered. The whole amount of potassium was later excreted, although the excretion occurred some days after the end of the period of negative nitrogen balance. There was also retention of phosphorus during globin administration. Although there did not seem to be any later excretion of phosphorus, irregularities in the balance figures may have masked such changes.

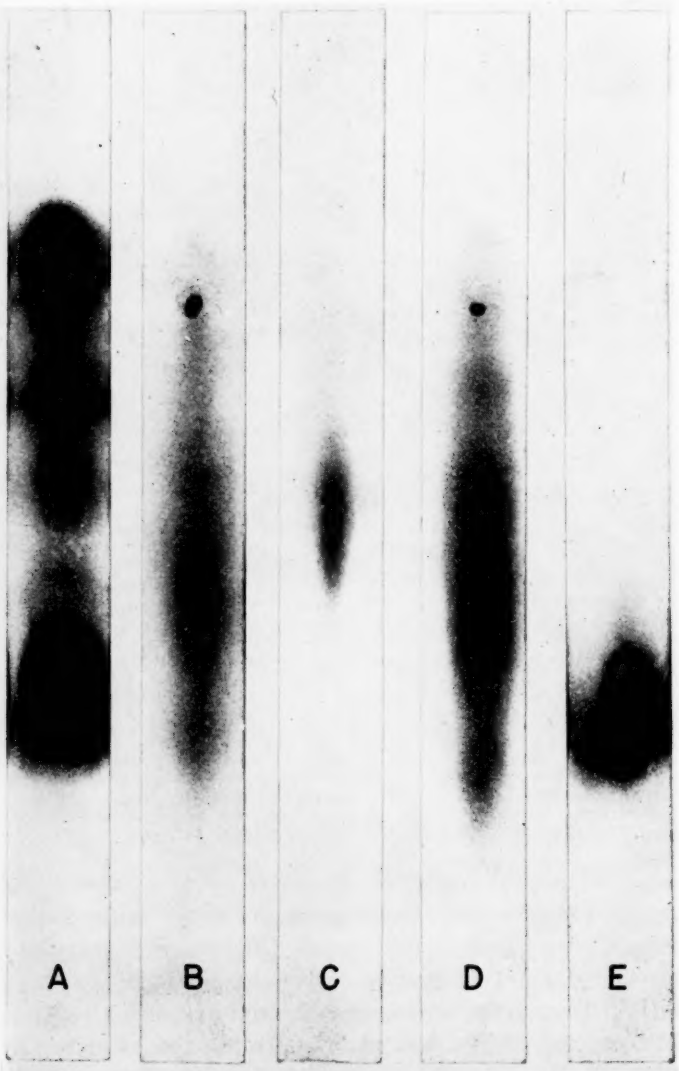

Fig. 3.—Filter paper electrophoresis patterns. All patterns were run at the same time under the same conditions. The dark spots at the upper end of patterns *B* and *D* indicate the level of application of the protein solutions to all strips. *A*, Normal human serum. From below upward the spots represent albumin, alpha-1, alpha-2, beta and gamma globulin. *B*, Urine protein from patient W. P. during globin administration. *C*, Modified human globin alone. *D*, Urine protein from patient F. S. during globin administration. *E*, Bovine serum albumin.

DISCUSSION

All the clinical reactions from modified human globin described here have been reported previously but not in such marked form. The appearance of reactions seemed to be related to the speed of infusion of globin. The rapid infusion of an 8 per cent solution gave marked reactions, while 4 per cent globin, either in 5 per cent dextrose solution or in distilled water, administered slowly gave less severe, but similar, reactions.

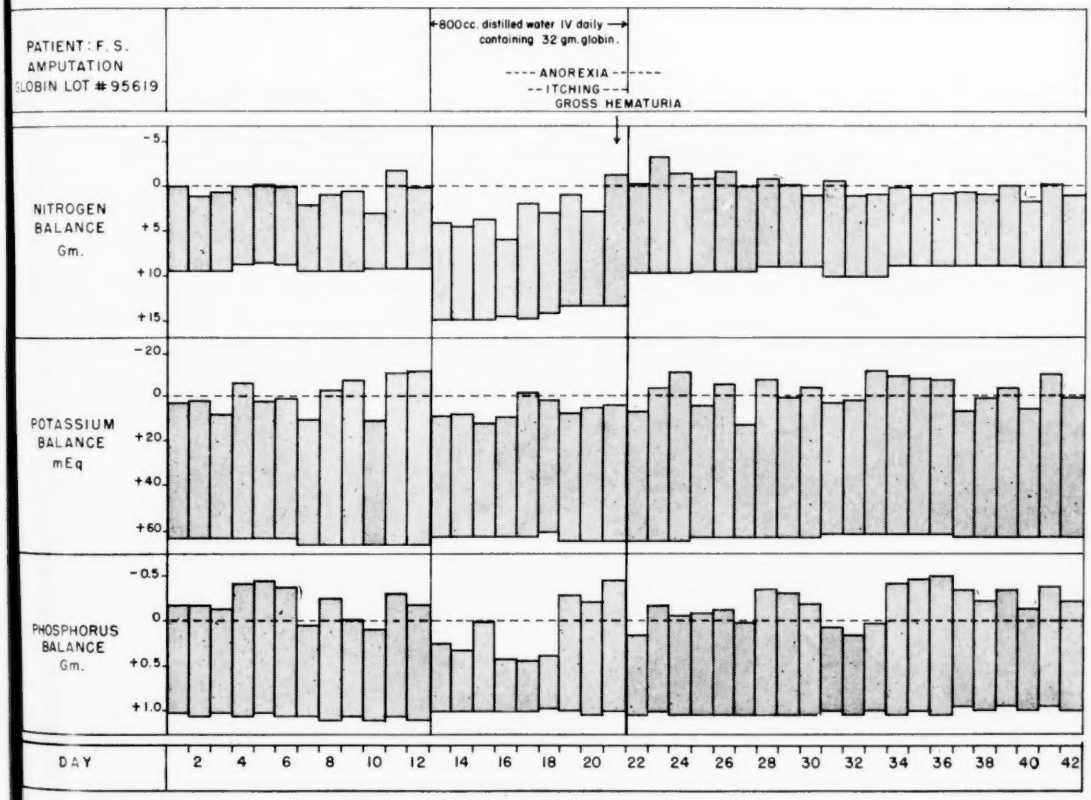

Fig. 4.—Balances of nitrogen, potassium, and phosphorus before, during, and after globin administration.

In the rapid infusion studies, globin produced an immediate fall in the serum albumin, a rise in the serum globulin, and a rise in the hematocrit. Dilution of the plasma by the globin infusion may have caused the fall in the serum albumin, while the rise in the serum globulin was probably due to the measurement of circulating globin in the globulin fraction. The rise in the hematocrit was presumably caused by a decrease in the plasma volume due to the escape of whole plasma from the vascular system. Measurements of the plasma volume by the dye method have shown increases after globin infusions equal to 50 to 100 per cent of the volume injected.[3] Measurements calculated from the hematocrit and red cell volume determined with red cells tagged

TABLE II. TOTAL BALANCES OF NITROGEN, POTASSIUM AND PHOSPHORUS DURING ADMINISTRATION OF MODIFIED HUMAN GLOBIN CALCULATED AS DEVIATIONS FROM CONTROL BALANCES (PATIENT F. S.)

PERIOD DAYS	CONTROL 1-12	GLOBIN 13-21	CONTROL 22-27	CONTROL 28-36	CONTROL 37-42
Nitrogen (Gm.)	0	+21.0	−9.5	− 1.3	+1.6
Potassium (meq.)	0	+50.8	−0.7	−45.0	−0.1
Phosphorus (Gm.)	0	+ 2.66	+0.92	− 0.23	−0.40

with P^{32} have shown that the plasma volume increase after globin is only 25 per cent of the volume injected.[15] An actual decrease in the plasma volume after globin infusion has not been reported.

The patients who received repeated infusions of globin showed a rise in the serum globulin which was not due to circulating globin. This rise was in the gamma globulin, not in the alpha-2 or beta globulin where globin should appear. The significance of this rise in gamma globulin, and of the rise in erythrocyte sedimentation rate, is unknown, although it is suggestive of a nonspecific response of the reticuloendothelial system.

The gravest consequences of globin administration were the changes in renal function. The appearance of red cells, white cells, and casts in the urine, even after a single infusion of globin, indicates the production of an inflammatory reaction in the kidney. In one patient this reaction proceeded to the point of gross hematuria. It has been reported[16, 17] that the clearances of inulin and para-aminohippuric acid are not changed by single infusions of globin. Creatinine clearances did not change in patient R. LaP. during a single large infusion of globin, but did fall gradually in patient W. P. after repeated infusions. Fortunately all the reactions reported here were completely reversible.

The data from the balance study show that only about one-fourth of the administered globin nitrogen was retained. During the period of globin administration potassium was retained in the proportion to nitrogen that would be expected if protoplasm were being formed (2.7 meq. potassium per gram of nitrogen[18]). Since all this potassium was later excreted, it is unlikely that a significant gain in tissue protein resulted. It is more plausible that the retained globin was stored in the body unchanged. These data are in essential agreement with those of others.[10, 11]

The toxic reactions from the intravenous administration of modified human globin indicate that the material in its present form is not suitable for use as a plasma substitute. The observed hemoconcentration and renal changes are highly undesirable in the treatment of shock. In addition, the anorexia produced by repeated infusions of globin is undesirable in the management of undernutrition and hypoproteinemia.

SUMMARY

Single rapid infusions of modified human globin produce reactions manifested by erythema, pruritus, urticaria, fever, hemoconcentration, and an increase in red cells, white cells, and casts in the urine.

Repeated slow infusions result in anorexia, an increase in the serum gamma globulin, elevation of the erythrocyte sedimentation rate, and an increase in the red cells, white cells, and casts in the urine which may progress to gross hematuria.

In one patient studied by balance methods only about one-fourth of the administered globin was retained in the body; there is evidence to suggest that this amount was not metabolized but was stored unchanged.

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A STUDY OF ANTIBODY FORMATION IN PATIENTS WITH MALIGNANT LYMPHOMAS

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IT HAS been a clinical impression for many years that patients with malignant lymphomas, especially Hodgkin's disease, are poor antibody formers. Dubin[1] has termed this state as a poverty of antibody response.

Parker and associates[2] in 1932 demonstrated that patients with advanced Hodgkin's disease frequently fail to react to the tuberculin skin test. This anergy to tuberculin has been noted also in patients with active pulmonary tuberculosis.[3] Subsequent studies have documented well these observations.[4, 5] Wise and associates,[6] studying patients with active brucellosis and Hodgkin's disease, were unable to demonstrate anitbodies to Brucella. Dubin[1] reported that with coexistent brucellosis and Hodgkin's disease, 14 of 24 patients had positive blood cultures for Brucella, yet only a very few had specific antibodies to Brucella.

Hoffman and Rottino[7] studied the antibody response of 13 patients with Hodgkin's disease who were immunized with a standard dose of Typhoid-Paratyphoid vaccine. Using a group of student nurses as controls, they were unable to demonstrate any significant difference in antibody response in the two groups. In the same study, they also demonstrated an inconstant effect of nitrogen mustard on the production of antityphoid agglutinin in a series of eight patients. Except for this study of Hoffman and Rottino, which utilized a serial dilution agglutination technique, there have been no quantitative studies on antibody response in patients with malignant lymphomas.

The purpose of this study was to determine the antibody response of patients with malignant lymphomas to a type-specific pneumococcus capsular polysaccharide.

MATERIALS, SUBJECTS, AND METHODS

Materials.—Type-specific pneumococcus capsular polysaccharide I was used as the antigen.† An isotonic saline solution containing 0.1 mg. per milliliter of type-specific polysaccharide I was prepared according to the method of Kabat and Mayer.[8] Two-tenths per cent phenol was added as a preservative.

Subjects.—Three groups of patients were studied.

Group I: This group, which served as the normal controls, consisted of 16 patients who had no known malignant disease and who were in good health. Only one patient (No. 2

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Read before Third Cancer Research Conference of Damon Runyon Memorial Fund Fellows, Oct. 10, 1952.

Received for publication, Nov. 28, 1952.

*Damon Runyon Clinical Cancer Research Fellow, 1951-1952.

†Kindly supplied by Dr. Frank Maltaner of the Divisions of Laboratories and Research, New York State Department of Health, Sloan-Kettering Institute for Cancer Research, New York, N. Y.

gave a history of hay fever. The average age was 43.2 years, with a range of 24 to 62 years. The sex distribution was 10 females and 6 males.

Group II: This group consisted of 14 patients (9 with lymphosarcoma and 5 with Hodgkin's disease), who were in good health with no signs of active disease, and who had received no therapy for a three-month period prior to the onset of this study, and who had received no therapy for the duration of the study. The average age was 45.5 years, with a range of 18 to 68 years. The sex distribution was five females and nine males. These were unselected cases, the only prerequisites being clinical absence of active disease, no recent therapy, and the willingness to participate in the study.

Group III: This group consisted of 10 hospitalized patients (6 with Hodgkin's disease and 4 with lymphosarcoma), who had active disease and who were initially treated with a total dose of 0.4 mg. per kilogram of body weight of nitrogen mustard (Mustargen) one to two days prior to the injection of the type specific polysaccharide. The average age was 44 years, with a range of 21 to 70 years. The sex distribution was 9 males and 1 female. Because of the severity of the disease many patients received additional therapy in the form of local irradiation, steroids (cortisone acetate), or triethylene melamine (TEM) before the completion of the study.

Methods.—All patients received one injection of 0.1 mg. of type-specific pneumococcal capsular polysaccharide I (S_1) subcutaneously. Nitrogen mustard was administered to those patients in Group III, twenty-four to forty-eight hours before the injection of S_1. Blood was drawn prior to the injection of S_1 and at two- and six-week intervals after the injection. The sera were immediately separated and kept in the frozen state until analyzed. Sterile technique was employed in obtaining the blood and in the handling of the sera, but 1 per cent by volume of 1 per cent merthiolate solution was added to each serum prior to freezing as an additional precaution.

Human complement was removed by the method of Heidelberger and associates[9] which utilizes an egg-albumin antiegg-albumin precipitation. Aliquot portions of the complement-free sera were set up as follows, in sterile test tubes: four 1 ml. portions were measured out, one serving as a blank. To the other three, 0.05 mg. of S_1, in 1 ml. borate buffer solution (pH 8) was added. The contents were thoroughly mixed. After capping with sterile rubber caps the tubes were kept at room temperature for thirty minutes and then placed in the cold room for one week, with occasional twirling. The precipitates were washed three times with cold saline, and the quantitative analyses were carried out using Pressman's modification of the Folin-Ciocalteau method.[10]

TABLE I. ANTIBODY FORMATION IN NORMAL HUMAN BEINGS

SUBJECT	MICROGRAMS OF ANTIBODY PROTEIN PER MILLILITER OF SERUM		
	PRE-IMMUNIZATION	POST IMMUNIZATION	
		TWO WEEKS	SIX WEEKS
1 (H. P.)	0	11	7
2 (M. H.)	8	210	
3 (W. M. S.)	1	32	60
4 (H. N.)	0	0	12
6 (R. J.)	0*	0	18
7 (N. A.)	14*	113	112
8 (J. F.)	0		410
9 (D. H.)	0	0	0
10 (C. G.)	0	0	0
11 (L. B.)	0	49	4
13 (E. B.)	0	34*	15
14 (J. H.)	18*	88	124*
15 (B. K.)	0	228	0
16 (M. P.)	3	6	6
17 (H. G.)	0	0	0
18 (S. G.)	0	24	0

*Indicates that only two sera were used in obtaining result.
Patients No. 5 and No. 12 failed to return for follow-up visits and, therefore, are not listed.

TABLE II. ANTIBODY FORMATION IN UNTREATED MALIGNANT LYMPHOMA PATIENTS

SUBJECT	DISEASE	MICROGRAMS OF ANTIBODY PROTEIN PER MILLILITER OF SERUM		
		PRE-IMMUNIZATION	POST IMMUNIZATION	
			TWO WEEKS	SIX WEEKS
0 (M. L.)	LSA	0	0	8
1 (A. F.)	LSA	0		0*
2 (M. S.)	HD	15*	7	18
3 (H. O.)	LSA	9	0	0
4 (M. Z.)	HD	0	8	
5 (E. S.)	LSA	11	20*	24
6 (B. B.)	HD	0	129*	10
7 (S. L.)	HD	0	0	0
8 (C. F.)	LSA	0	35	0
9 (L. C.)	LSA	0	0	0
10 (E. G.)	LSA	13	4	28
11 (U. D.)	HD	0	3	0
12 (I. W.)	LSA	0	0	0
14 (A. W.)	LSA	0	0	0*

*Indicates that only two sera were used in obtaining result.
LSA, abbreviation for lymphosarcoma.
HD, abbreviation for Hodgkin's disease.
Patient No. 13 failed to return for follow-up visit and, therefore, is not listed.

RESULTS

Table I lists the results of immunizations in Group I, the normal control group. Results are expressed in micrograms antibody protein per milliliter serum. Values below 20 micrograms per milliliter were interpreted as being insignificant because of the limitations of the method. Nine of the 16 patients formed a significant antibody titer. One patient (No. 6) had a borderline value of 18 micrograms antibody protein per milliliter serum.

Table II lists the titers obtained in Group II, the untreated malignant lymphoma control group. Only two patients (No. 6 and No. 8) developed a significant antibody titer. The results in patients No. 2, 5, 10 are difficult to interpret because of the high preimmunization titer. Since the difference between this level and the highest obtained after immunization is less than 20 micrograms of antibody protein per milliliter serum, these are considered to be insignificant. Therefore, 2 of 14 patients (14 per cent) developed a significant titer.

Table III lists the results obtained in Group III, the treated malignant lymphoma group. These data are difficult to interpret because many received the additional therapy as noted. Of the 10 patients studied, two (Nos. 7 and 8) developed a significant titer. This finding is about the same as in Group II. It is of interest that there is no correlation between antibody level and the total white blood count.

Combining Groups II and III, a total of 24 patients with malignant lymphoma, it is noted that only 4 or 17 per cent developed a significant antibody response to S_1.

COMMENTS

These findings provide quantitative data to substantiate the concept of poverty of antibody response seen in patients with malignant lymphomas.

TABLE III. ANTIBODY FORMATION IN TREATED MALIGNANT LYMPHOMA PATIENTS

SUBJECT	DISEASE	A	W.B.C. (THOUSAND)	B	W.B.C. (THOUSAND)	C	W.B.C. (THOUSAND)	ADDITIONAL THERAPY COMMENT
1 (S. V.)	LSA	0	5.4	0	3.9	Died		X-ray to neck and groin—TEM
2 (H. S.)	HD	1	17.0	11*	5.4	10	7.2	X-ray to spleen, Cortisone, TEM
3 (C. T.)	HD	0	5.7	0*		0*		
5 (R. H.)	LSA	0	3.7	7	4.0	0		X-ray to chest
7 (D. B.)	HD	0	3.0	81	2.8	22	2.5	Died within two months
8 (M. M.)	LSA	6	5.5	780	3.3	Died		
9 (D. R.)	HD	0	7.2	0	3.2	0	16.0	X-ray to chest, HN_2
10 (J. R. C.)	HD	0	9.3	0	1.4			X-ray to abdomen
11 (L. C.)	LSA	8	7.0	6	2.3	0	4.0	
12 (J. C.)	HD	15	8.0			4	6.4	

Column A—Preimmunization titer in microgram antibody protein per milliliter of serum.
Column B—Two weeks post immunization titer in microgram antibody protein per milliliter serum.
Column C—Six weeks post immunization titer in microgram antibody protein per milliliter serum.
LSA—Abbreviation for lymphosarcoma.
HD—Abbreviation for Hodgkin's disease.
*Indicates that only two sera were used in obtaining result.
Patients Nos. 0, 4, and 6 died within two weeks after injection of antigen and therefore are not listed.

There is, however, little in the data to explain these findings. Cannon[11] reported that low protein diets would impair antibody formation in the rat. The patients in the untreated control group of malignant lymphoma (Group II) were in good health and showed no clinical signs of active disease. Many of the treated group (Group III) were in a debilitated and in a preterminal condition. In view of the study of Balch,[12] however, who noted that severely ill, nutritionally depleted patients were capable of producing antibodies to diphtheria toxoid as well as or better than the healthy controls, one cannot invoke this concept to explain the anergy. It is also unlikely that the impairment of antibody formation is due to a pyridoxine deficiency, as noted by Stoerk and co-workers[13] in rats, since the hospitalized patients received multiple vitamin supplements with their diet.

As this disease involves the reticuloendothelial (RE) system, it has been postulated that the replacement of the RE system with diseased tissue is a possible explanation for the poor antibody production. Although it is generally accepted that the RE system plays an important role in antibody formation, the exact nature of this is not well understood. A question exists whether the RE system is the site of antibody synthesis or merely a means of modifying circulating antigens (scavenger role) and allowing the altered antigens to go to other tissues where antibodies are formed. The recent work of Dixion,[14] wherein he noted no alteration of antibody formation when total body irradiation, in a dose large enough to depress the bone marrow and lymphoid tissue of a rabbit, was administered after the injection of the antigen (bovine gamma globulin) suggests that these elements are not essential for antibody synthesis. That there may be other areas or mechanisms of antibody formation is also suggested by patient No. 8 in Table III who had extensive disease, de-

veloped a neutropenia following nitrogen mustard, died from this disease, and yet who was able to develop a titer of 780 micrograms antibody protein per milliliter serum, within two weeks after the injection of S_1.

The effect of nitrogen mustard therapy in such patients is of considerable interest, since it has been well established that this agent will depress the formation of or delay the appearance of antibodies.[14-17] As most of the hospitalized patients were seriously ill, many required additional therapy, especially irradiation, which is also known to depress antibody formation.[18] Because of the small number of patients studied, one cannot say whether or not the nitrogen mustard caused a further depression of antibodies. It would appear likely, however, that the therapeutic amount of nitrogen mustard used would not have such an effect since one needs several doses of 1 mg. per kilogram of body weight of this drug in the experimental animal to inhibit antibody production.[17]

SUMMARY

1. A quantitative study of the antibody response of patients with malignant lymphomas to type-specific pneumococcus capsular polysaccharide I confirms the concept of poverty of antibody response seen in such patients.

2. 62 per cent of the patients in the normal control group (Group I) formed significant titers of antibody to S_1. Patients with malignant lymphoma formed significant antibody titers in 14 per cent of the untreated group (Group II) and in 20 per cent of the treated group (Group III).

3. No conclusions could be drawn about the effect of nitrogen mustard on antibody formation in patients with malignant lymphoma.

The author is indebted to Dr. David Pressman for advice, to Mr. Rubin Medina for technical assistance, and to Dr. Lloyd F. Carver for permission to study the patients in the Lymphoma Clinic and on the Lymphoma Service of Memorial Center.

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THE RELATION OF ECZEMA TO ATTITUDE AND TO VASCULAR REACTIONS OF THE HUMAN SKIN

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THE disease characterized by the appearance of itchy, red, thickened areas on the skin, sometimes lichenified and sometimes weeping, has been variously called eczema, atopic dermatitis, or neurodermatitis, among other names, depending in part upon the distribution of the lesions, the age of the patient, and the presumed cause of the eruption.[2] The present study was designed to explore the relation of such lesions to vasomotor changes in the skin and to correlate changes in them with the prevailing life situations, emotions, and attitudes of the patient. The significance of these in this disease, which for convenience will be chiefly referred to in this paper simply as "eczema," has been discussed by many authors.[1, 11, 15, 17, 18, 19, 22, 24]

SUBJECTS

Thirty-one patients who had applied to the Out-Patient Department of the New York Hospital for treatment of eczema were the subjects of the study. Nine were men and twenty-two were women. Their ages ranged from 17 to 75 years. Some had been seen earlier in the Dermatology, Allergy, or other clinics; others were referred directly from the admitting room without inquiry into possible etiological factors. All of these more or less randomly selected patients were included in the study.

The site of the dermatitis varied from patient to patient. There were three men whose lesions were on the scrotum, perineum, and inner surface of the thighs. Three of the women had lesions only on the hands or feet at the time they were seen, although one of these had at one time had much more generalized involvement. One woman had a lesion confined to the back of the neck, and two others had involvement only of the periorbital region. The other patients all had fairly widespread disease involving the face and the extremities, particularly the flexor surfaces.

PROCEDURE

Detailed life histories were obtained from all patients, with special reference to the correlation of significant events in their lives with the onset of their illness, and with exacerbations and remissions in its course. Possible relations of flare-ups to exposure to foods or other allergens were also carefully sought for.

Experimental interviews were undertaken with fifteen of the group. These were carried out only after considerable information concerning the patient's life and his reaction to stressful life situations had been gathered in at least four sessions of interviewing, each lasting an hour or more. An experimental interview began with an introductory control period during which neutral or pleasant topics were discussed, followed by the deliberate introduction of topics known to have been associated with exacerbations of the disease. The interview concluded with a period of diversion and reassurance.

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Aided by grants from the Commonwealth Fund.
Received for publication, Feb. 4, 1953.

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During experimental interviews, two tests of cutaneous vascular function were performed at five- to ten-minute intervals. One test was measurement of the skin temperature by the Hardy radiometer.[12] This measurement reflects the contractile state of the arterioles, which, as the site of most of the peripheral resistance, control the cutaneous blood flow, and hence determine the hue of the skin—blueness or redness, and also skin temperature. The tone of the minute vessels (the capillaries and venules)[16] which determine the intensity of skin color was estimated from the reactive hyperemia threshold determined according to the method of Di Palma, Reynolds, and Foster.[3] The reactive hyperemia threshold does not measure the actual caliber of the vessels, but rather their resistance to dilatation. It consists of applying a weight to the skin long enough to produce, after it is removed, an increase in skin color, i.e., a zone of erythema due to minute vessel dilatation. If the threshold is high it indicates relatively high minute vessel tone; if it is low, the minute vessel tone is relatively low. All measurements were made on the volar surface of the forearm. The experiments were carried out at room temperatures varying between 23° and 28° C. The temperature did not vary more than 1.5° C. during any single experiment.

During the discussion a special effort was made to determine the patient's *attitude* toward the event in question. In other words, he was asked to state (1) what his position in the situation seemed to be, or what he felt was happening to him, and (2) what action he wanted to take. It was also ascertained that his attitude during the experiment was the same as that which he had developed at the time the disturbing event actually occurred.

RESULTS

Relation Between Life Situations and Symptoms.—In 26 of the 31 patients there was established a clear-cut temporal correlation between life events which the patient recognized as emotionally disturbing and exacerbations of the illness. The correlation applied not only to major attacks, but also to relatively minor episodes of itching and scratching lasting for only an hour or two. In 20 of this group the onset of eczema could also be correlated with highly significant events; the other six had had symptoms almost constantly since early childhood, and the exact circumstance surrounding their beginning could not reconstructed.

Of the five patients in whom correlations could not be established, three had obviously severe difficulties in their life situations, but discontinued attendance at the clinic after two or three visits. The other two were almost wholly uncommunicative and also dropped out of the clinic after three and four visits, respectively.

Concerning contact with potential allergens, one man noted increased difficulty in exposed areas of skin while he was working in a dusty environment. One woman said that eggs made her eyes "all black and horrible," although it was carefully established that her exacerbations of eczema did not correlate with the ingestion of eggs. Another woman, who said that eating nuts increased the itchiness of her skin, could not be induced to try them during the period of study. Nevertheless, she had many exacerbations and remissions of her dermatitis during this period. In none of the remaining patients was there even suggestive evidence of a relation between eczema and foods or contactants.

Attitude Toward Traumatic Situations.—Although the life situations which were correlated with attacks of eczema varied in type, the patients' attitudes at such times were essentially similar. In these situations they considered them-

selves to be *frustrated*. That is, each one felt that there was something he wanted to do, that he was prevented from doing it, and that action on his part to deal with the interfering circumstances was essentially useless or impossible. He was preoccupied with the interference or blocking, and *not* with carrying out the desired action.

As a special case of this, some patients occasionally had mild attacks of eczema in circumstances usually called "embarrassing." The word "embarrass" "implies some influence that impedes thought, speech or action, and may be used with reference not only to persons but to the things they plan or desire to do" (Webster's New Collegiate Dictionary, 1945), and one prominent feature of such situations is the inability to think of anything to do.

Patients did not necessarily feel frustrated in connection with the stressful situation at all times, but they always felt so at the time they were having the symptoms referable to dermatitis. Feelings of hostility (that is, of plans for, or wishes to take, aggressive action) were never associated with attacks of dermatitis.

It should be noted also that *anxiety* and *tension* were not associated with exacerbations of eczema, if these terms are strictly defined as meaning, respectively, the feeling that something bad is about to happen, and the desire to take some kind of action. It is true that patients in this series often developed anxiety, but at those times their skins did not itch. It was only if the anxiety was succeeded by feelings of frustration that exacerbations of dermatitis developed. This was demonstrated from life history material on many occasions, and is explained by the experimental finding that vasoconstriction, not vasodilatation, accompanies feelings of anxiety.[11] Unmixed feelings of depression and hopelessness were likewise not associated with attacks of dermatitis.

It was possible in some instances to understand, in terms of the patient's attitude, reasons for the localization of the lesions. In general, predominance of difficulty on the hands was correlated with frustrated efforts to induce someone else to do something. Thus, one girl had trouble with her hands when she was unable to make her fiancé be sufficiently ambitious in business; another woman had flare-ups on her hands when she failed to prevail on her husband to help her with the housework; still another when her father refused to take as firm a stand with his tenants as she thought desirable.

When the face was principally involved, however, the problem usually revolved around the position of the patient in the eyes of others. The first woman mentioned in the preceding paragraph illustrated this. When she felt that she was not understood or was unable to obtain from others the esteem which she wished, the lesions on her face flared up, without much change in the hands.

Two of the three men with scrotal lesions were involved in sexual conflicts. In one of the two women whose lesions appeared first around the eyes, there was a close correlation between exacerbations and a desire to weep, which was inhibited because of disapproval of family members.

Vascular Changes During Stressful Interviews.—It was immediately obvious that in discussions of life stresses there was flushing of the skin, accompanied

by increased itching and scratching, which suggested that significant physiological changes involved in the production of lesions took place in the blood vessels.

In order to define these changes more precisely, a total of 19 experimental interviews was performed with 16 subjects. In 8 of these patients skin temperature and minute vessel tone were followed concomitantly; in 7 others skin damage was so great that the reactive hyperemia threshold could not be obtained, and only skin temperature was measured. In one patient only the reactive hyperemia threshold was followed.

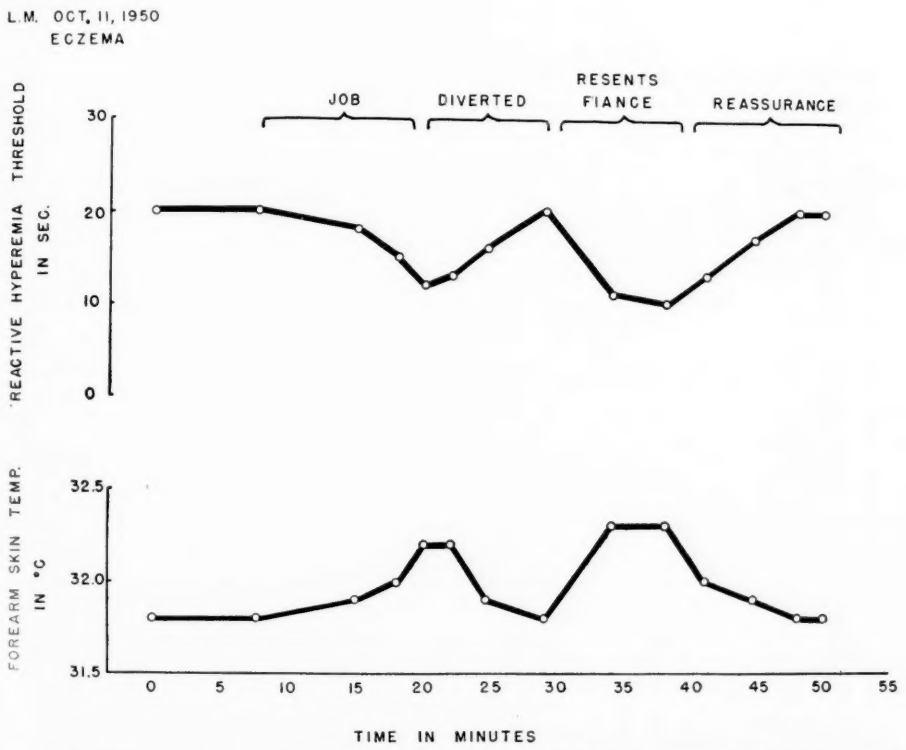

Fig. 1.—Cutaneous vasodilatation, involving both the minute vessels and the arterioles, on the forearm skin of L. M. (Case 3) during experimental interview. Exacerbations of her eczema were found to occur in response to certain features of her job and to some aspects of her fiancé's behavior. It will be seen that mention of each of these subjects provoked vascular changes.

In 15 of the experiments, on 14 subjects, there was increase in skin temperature of at least 0.3 degree (indicating dilatation of arterioles) when the subjects were discussing those events in their lives known to have been associated with exacerbations of the dermatitis. In the 8 of these 15 experiments in which it was technically possible to measure the active hyperemia threshold, and in one additional experiment, there was a decreased minute vessel tone, indicated by a fall in the threshold of at least five seconds. When these changes were marked, the subjects complained of itching and often scratched. These changes

occurred only when the discussion aroused in the patient the same attitude of frustration which he had developed at the time of the actual occurrence of the events.

Even if "spontaneous" itching did not occur, light scratching of the skin by a pin was felt as itchier than the same scratching during control periods of greater tranquillity, in which there was no cutaneous vasodilatation. Typical interviews are illustrated in Figs. 1, 3, and 4.

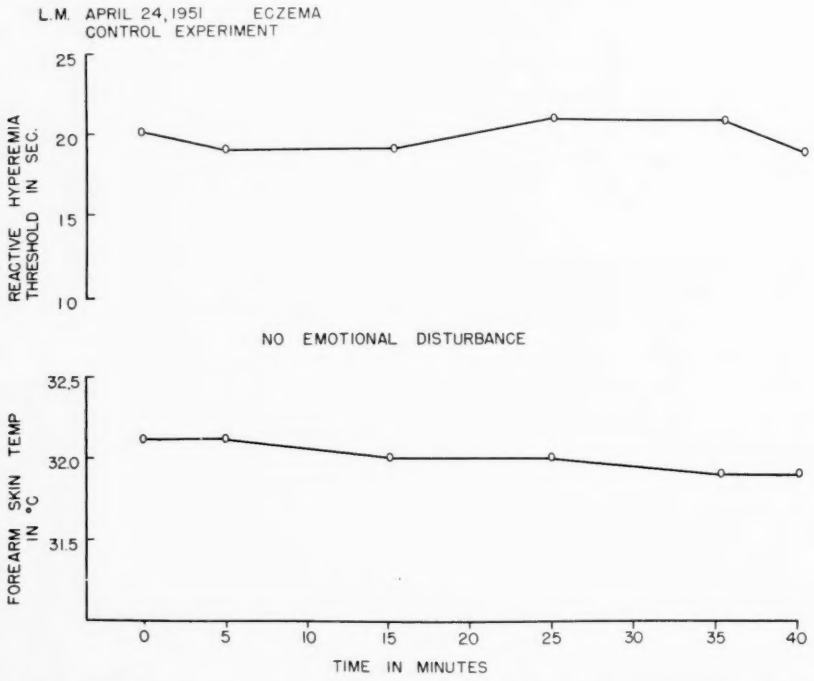

Fig. 2.—Lack of cutaneous vascular change in L. M. during interview conducted in a reassuring way, with avoidance of stressful topics. The patient said that she was aware of no emotional disturbance on this occasion.

Illustrated in Fig. 2 is an experiment to show that with the deliberate avoidance of disturbing topics during an interview, significant cutaneous vascular changes did not occur.

Five of the patients developed during an interview feelings of hopelessness, with or without weeping. At these times there was no flushing, no increase in itching, and the skin grew cooler, not hotter, than it had been, even though there was a decrease in minute vessel tone.

Itching and Scratching.—Itching and scratching are such prominent manifestations in patients with eczema that they deserve special consideration. Their mutual relations have been described variously. It has sometimes been suggested that scratching is the primary phenomenon, with itching developing only as a result of the scratching or other manipulation. It has even been

proposed that the skin in this disease does not really itch at all, and that the patients scratch because they develop a feeling that the skin "needs" to be scratched.

Nature and occurrence of itching in the patients: Twenty-five of the patients were closely questioned about the nature of the experience they reported as "itching." They all stated that it was qualitatively exactly like that which follows a mosquito bite, and that it provoked the desire to scratch. They were

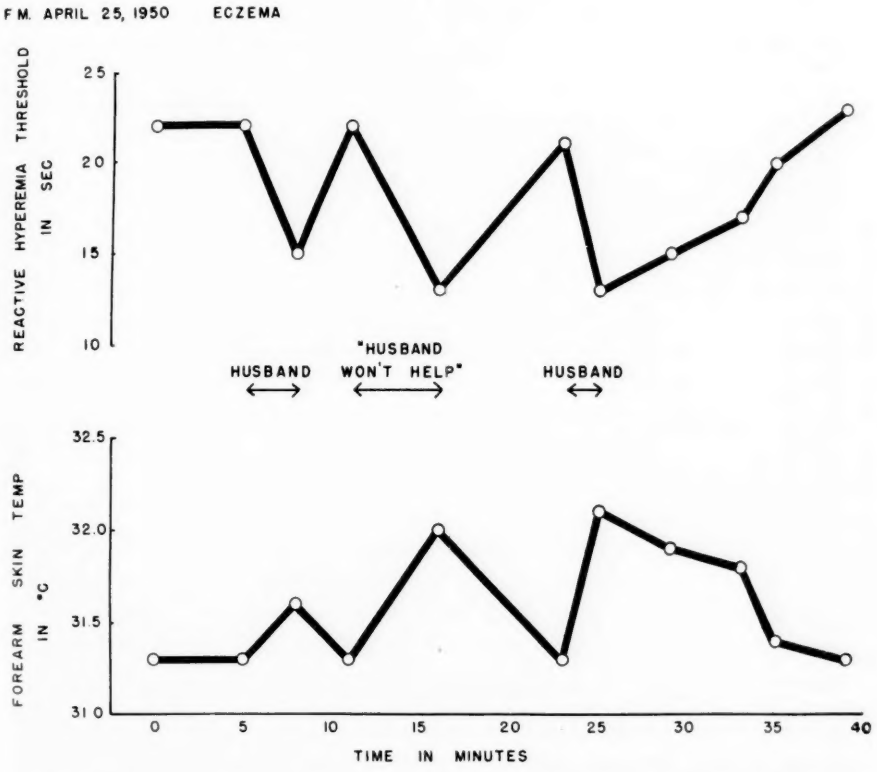

Fig. 3.—Cutaneous vascular changes on the volar surface of the forearm of F. M. (Case 4) during experimental interview. Each time her husband was mentioned, there was a fall in the reactive hyperemia threshold and a rise in skin temperature, indicating decreased minute vessel tone combined with arteriolar dilatation.

all also aware that it was much intensified at times when their skins felt hot, even on those occasions on which they consciously refrained from scratching. This association of itching with vasodilatation was confirmed experimentally (see below).

It therefore appears that scratching was not a necessary precursor of itching in these patients, any more than it is in individuals without eczema.

Effect of scratching on itching: Intense scratching usually relieved itching temporarily in these patients, as it does in persons without eczema. Painful

stimulation inhibits itching for a period of time outlasting the duration of the pain, and itching cannot occur in the hyperalgesic skin surrounding such an area of damage as is likely to be produced by severe scratching.[8]

On the other, mild scratching or other manipulation of the skin often intensified itching, or set it off if it had not been present. Furthermore, brief relief after scratching was likely to be followed by even more intense itching.

Reasons for scratching: Scratching by patients with eczema cannot, however, be simply explained as an effort to relieve itching, as many authors have noted.

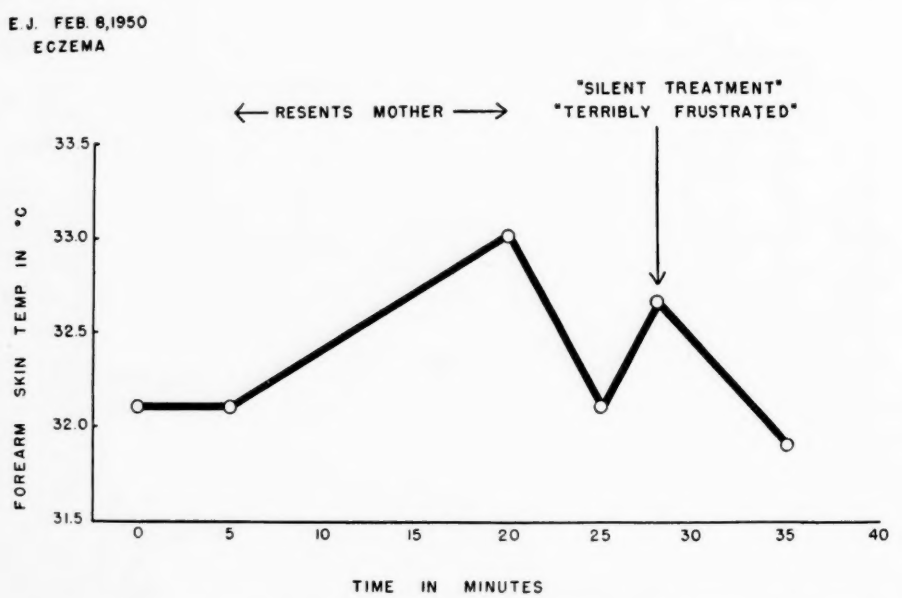

Fig. 4.—Interview with adult woman with lifelong eczema and asthma. The reactive hyperemia threshold could not be measured because of skin damage. Discussion of her mother's treatment of her when she was a child resulted in arteriolar dilatation.

Self-punishment was in many of the patients a very important element in scratching, so that the latter was much more intense and prolonged than seemed to be appropriate to the intensity of the itching. This aspect seemed to be of major significance in at least half of the patients, and was of some importance in all of them. Their attitudes were expressed by such statements as "I scratch until I've punished myself enough," "I take it out on myself," and "I stop when I've done myself enough damage."

There is, in addition, a definitely pleasurable aspect of itching and scratching on which several authors have commented. Two of the patients in the present series mentioned this as important. One woman said, "When I'm scratching, when the itching has ceased, it's the biggest thrill I could encounter," and also remarked that she was "more interested" in her itching than in sexual intercourse. One man referred to the "diabolical pleasure" he got from scratching.

Finally, many patients stated that they scratched sometimes when there was no itching, at times of "tension." In such circumstances it seemed to be comparable to table-tapping or any other habitual tension-relieving activity in individuals without skin disease.

In summary, then, scratching occurred in this disease because it relieved itching, because of a need for self-punishment, because it was a source of pleasure, and as an habitual manifestation of tension.

General Aspects of Personality.—No attempt was made to delineate with precision the personality characteristics of the patients in this study except for their development of the particular attitude described previously. (Efforts in this direction by means of psychological tests have been made by others,[1] with some success.) Certain features, however, deserve mention.

The women who developed the disease as adults were quite meticulous and preoccupied with cleanliness, neatness, and order. In this respect, however, they did not seem different from many women with disorders other than eczema.

Of the five adult men in the group, two were almost completely impotent. One of the others was unmarried and did not engage in sexual activity, and the other two seemed to have adequate sexual function. Eleven of the women were married and living with their husbands at the time of the study. Three of these consistently derived significant satisfaction from sexual intercourse, and seven of the others found it pleasurable at least occasionally. Only one approached complete frigidity.

Exhibitionism is occasionally mentioned as important in the genesis of dermatitis. There was no indication that members of this group had ever engaged in overt sexual exhibitionism, or had had conscious wishes to do so. Further, there was very little indirect evidence of exhibitionistic trends. The women, for instance, did not seem more concerned with clothes or other features of personal appearance than would be expected from cultural norms.

There was in all members of the group who had very visible lesions, especially on the face, a good deal of preoccupation with how they looked in the eyes of others, but it is impossible to be sure that this was not in large part a consequence of the lesions. It became less prominent whenever the skin improved, even if the improvement was brought about by such things as hospitalization on a medical ward or the administration of ACTH. There was, however, one man who was much preoccupied with feelings of physical inadequacy, focused particularly on the size of his genitals, and who had a good many attacks of flushing and itching when he felt that he was the object of the attention of others. Two adolescents in the group also were likely to have difficulty when "embarrassed" socially, but this did not seem much out of proportion to the same response in the average adolescent, and it may well be that it was only because of the pre-existing skin trouble that additional flushing resulted in symptoms.

Every patient who was seen for more than four times revealed fairly serious feelings of depression and hopelessness. Only one patient, however, actually

made a suicidal attempt, and she had the most widespread and most intractable skin involvement in the group studied. Part of the depression in many of the cases revolved around the skin lesions themselves, and the apparent impossibility of ever being cured. This, however, was not the whole story, and it was often contributed to by feelings of guilt about various things, or by a general sense of personal inadequacy. Many of them knew that their scratching had a great deal to do with producing the actual lesions, a point which their families frequently stressed, and felt guilty or inadequate because of their inability to stop scratching.

It was noteworthy, however, that the periods of most severe depression were not the times of most difficulty with the skin. When the patients became overwhelmed with feelings of hopelessness and despair, either in connection with the skin condition itself or for some other reason, they did *not* itch. It was not necessary for free weeping to be associated with the depression for the remission of symptoms to occur. This finding from the life-history material was confirmed and explained by the experimental finding in these and other patients that depression and despair are associated with cooling of the skin, not warming.[10]

Early Life History.—In the present study methods of obtaining and recording the data which make some degree of precision possible were not employed. There were, however, some things which recurred in many of the life histories and which seemed to be different in this group from the histories of patients with other disorders.

There was one feature of the early parent-child relationship which seemed to be of particular importance to those individuals who had eczema in childhood. This was the adoption by one or both parents of the "silent treatment" as a favorite method of discipline, so that the child who had transgressed against regulations was unable to get from the parent more than a grudging word or two for a period of hours or days. This was reported by 7 of the 14 patients who were young enough to have clear memories of their childhoods and who were studied intensively enough to give a reasonably good picture of their early lives. It was remembered by those patients who reported it as an intensely frustrating experience, and one with which they felt absolutely powerless to deal, since pleas for forgiveness or efforts to gain attention were all met with the same lack of response from the parent.

Ten of the patients, perhaps at least in part as a result of this special kind of treatment, found themselves in the relation to the parent (mother in all but one instance) described by Kepecs and associates[13] as "hostile-dependent." That is to say, they found themselves unable to take effective action for themselves, were unable to do without the support and approval of the mother, yet resented this situation and the person who had manipulated them into it. In one woman this conflict was still active even at the age of 41 years.

It seemed that the fundamental problem in many cases was that the mother had consistently used as a preferred method of punishment the withholding of affection, at the same time arousing guilt in the child when he did anything of which she disapproved. There seemed in some instances to have been ma-

ternal overprotection and direct interference with the exploratory behavior by means of which the average child comes to understand and learn to control his environment. The result of such behavior was that the child was engaged in a constant struggle to gain his mother's affection, but was never wholly successful in doing so. It would be interesting to know why he continued the struggle instead of giving it up completely, and one might hazard a guess that it was because his efforts were at least sometimes successful. In any case, he then seemed to be attached to the mother by his failure to develop successful techniques for getting along in his environment. It seemed also as though receiving maternal love was a necessary first step toward dealing with the world, and that the child who didn't get it was doomed to persist indefinitely in his useless endeavors.

One consequence of this was that the recipient of such treatment, aware that he was unhappy and frustrated, but unable to locate the sources of his difficulty, developed a confused feeling that he was to blame. This was very often directly intensified by behavior of the mother, who developed direct verbal or more subtle behavioral methods of reproaching the child for his failure to behave as she wished. The resulting guilt plus dissatisfaction with himself seemed to be what lay behind the scratching.

CASE REPORTS

CASE 1.—K. L. was a 41-year-old white woman who had had dermatitis of the face, hands, and shins, of gradually increasing severity, for about five years. She also had moderately severe vasomotor rhinitis.

She was one of several children of Orthodox Jewish parents, in very difficult financial circumstances. Because of her responsibilities for the younger children, and because of her mother's generally restrictive attitudes, she said that she had never played at all. Her mother stressed "obedience and cleanness." Her father seems to have played a relatively small part in her life, and to have been somewhat derogated by her mother.

The patient was made to feel "very guilty" by her mother during much of her childhood. Her mother's usual method of punishment was to refuse to speak to the patient until "I got down on my knees and begged forgiveness." "I felt very guilty, I felt I harmed her greatly." She had a favorite aunt, however, to whom she turned for emotional support when she became too unhappy at home.

She married a man whom she did not love because her mother "made me feel I'd be an old maid." Her husband was a very unassertive civil servant, without particular ambition and sharing none of the patient's cultural and educational aspirations. She said, "Everything in our married life I've had to shoulder." Her dermatitis began at a time when their landlord was making efforts to evict them. Her husband failed to take any action, leaving the whole burden of resisting the landlord to her.

She had two daughters, the second of whom, Louise, had a congenital dislocation of the hip, which required a great deal of medical attention with several surgical procedures. The patient was for a time completely absorbed in this daughter's care, and was "cruel" to the older girl as a result. She remarked, "What's happening to Louise is happening to me."

Her sexual adjustment was never satisfactory, apparently partly because of feelings of guilt about sex originating in childhood, intensified by her feeling that she didn't love her husband enough, and by what she called an "affair" with another man after her marriage. This actually amounted only to her falling in love, without any direct sexual contact. In addition, she resented her husband's inadequacies in sexual activity.

Her skin cleared completely during a visit to a sister in another state, where she felt "so protected," but the dermatitis otherwise persisted, with exacerbations and remissions, from the time of its onset until she came to the hospital.

Conflict with her mother persisted throughout her life. Once, concerning her mother's offer to buy her a coat, she said, "It makes me feel good to think she loves me that much, but I don't like the sympathetic understanding—I like to stand on my own two feet." Also, "I feel small when I'm with her," and "The only way to get her attention is by being sick." "I feel that I want to be in her limelight all the time, and I hate feeling that way." The mother always took her husband's part in any dispute, and, in general, "I'm always wrong in the eyes of others."

Signs of increasing sexual interest on the part of the adolescent older daughter disturbed the patient. She was also disturbed because the family financial situation prevented sending the girl to college.

Flare-ups of eczema occurred in connection with difficulties in all of these areas of conflict. Her attitude toward any situation which provoked an exacerbation of the eczema was always some variation on the frustration theme: "I can't get him to do it," "I can't make her understand," "She won't listen to me," etc. She had signs and symptoms from time to time other than those associated with the skin disease, such as vascular headaches or rhinitis, occurring in association with different attitudes which she generated toward various stressful situations. Anxiety, by which is meant the feeling that something bad was likely to happen, was not uncommon, but was not directly associated with dermatitis. One day she began to itch while she was waiting for her daughter to return from the park. Questioned about the episode, she at first said that she was only worried about what might have happened to the girl. It then developed, however, that much of her preoccupation had been with her daughter's constant failure to heed her admonitions about such behavior, and that she felt unable to make any impression on the girl.

Of all the patients, she was the one who most clearly stated that she scratched when she felt "guilty" or "ashamed of myself" or wanted to "take it out on myself," and she often produced severe excoriations in addition to the lesions of eczema. She was unable to resist picking at these lesions, and they frequently became infected.

She was seen on 31 occasions over a period of eighteen months, and was considerably improved when last seen. The prognosis for maintaining the improvement did not seem very favorable, however, since she gave few indications of important modifications in her attitudes or in her way of dealing with problems.

CASE 2.—R. S. was a 21-year-old university student who had had severe generalized eczema since early childhood. He also had moderately severe hay fever.

His parents were both living. They had never got along well together, but had never actually separated, although the father was often gone for periods of several weeks on business trips. When the patient was born, his mother attempted to find in her relation with him everything she was missing with her husband. When she was interviewed (at her own request), she said, for instance, "When he was born we were very close—it was just like Siamese twins." She remarked also that he always, even as a small child, "kept himself nice and clean." She thought perhaps she had made too many decisions for him. "He just wasn't interested in making his own."

The patient said that she was "overprotective" and wanted to make him "more conscientious." His attitude toward her was ambivalent. He resented the fact that she "controlled" him, by making him feel guilty if he failed to do as she wished, and yet he always felt that "I wanted to take my father's place with my mother." He unequivocally disliked and resented his father, of whom he said, "You can't argue with him; I've never really stood up to him."

He had had a severe attack of eczema beginning about a year before he was first seen in the clinic and continuing without any prolonged improvement. The exacerbation began

while he was away at college, and the reasons for it were never fully clarified. It eventually led to his coming home and attending a local college. His skin failed to clear up, however, and he eventually sought further medical help.

In the course of treatment, he described many attacks of skin trouble occurring following arguments with either or both of his parents. He felt that they "didn't understand" and that he never really got his points across. He was never allowed to "express ((himself) enough, to do what I wanted." At college his skin bothered him when he was confronted by a test problem which "completely stumps me." In general, though, his skin was much better when he was at school than when he was at home. He also noticed that itching was temporarily relieved by masturbation.

At one time he made a visit of several months to a southern state, during which he was almost completely free of symptoms. After returning home, and finding his skin worse again, he took a room away from his parents' home and again improved.

He made a total of 22 visits to the clinic in a period of fourteen months, and was having considerably less trouble in the last half of this period than in the first. He had begun to "tell mother off" sometimes, and decided to follow a course of study which his parents were somewhat opposed to. He said that his attitude had changed; previously he had felt "Won't you please see it my way?" now "I don't need their approval." This change involved transferring to a university in another state, so that it was not possible to tell whether he would have maintained his improvement if he had remained in his home environment.

CASE 3.—L. M. was a 23-year-old woman who had had dermatitis of the face, hands, and flexural surfaces intermittently for eleven years. It had not become a serious problem until about three years before her first clinic visit. She also had occasional attacks of rhinitis.

She was the second of three children. Her mother had been the dominant figure in the home in her early childhood, with her father remaining "a pale shadow in the background." The mother characteristically grew quite agitated in dealing with minor daily problems, and at such times often rebuffed the patient. The patient said, "I got pretty much what I wanted but it tended to be what she wanted." When she attempted to do things at home, her mother often said such things as "Oh, you're hopeless—I have to do everything."

As she grew older, her father became a considerably more significant figure. He exerted pressure on her for certain kinds of achievement, including some forms of athletics, and literary composition. Her athletic performance never seemed to be quite satisfactory to him, however, and in her writing she felt that he "wasn't really interested in what I had to say." In general, then, she never quite got from him the rewards she wanted. Her mother she came to regard as not worthy of serious consideration, and resolved not to be like her in being upset by so many little things. She remembered "trying to keep up" with her father in walking, for instance, and her feeling of frustration at not being able to do so and at his lack of sympathy for her difficulty.

Following a difficult secondary school experience, in which she felt awkward, unpopular, and unable to achieve a place for herself among the other girls, she went to college, which she enjoyed. She was quite successful academically and socially, and had no difficulty with her skin during most of her four years there.

The first serious attack of dermatitis began while she was on a vacation trip with other students and was falsely suspected by an older man in charge of the group of having allowed a boy to stay in her room. She was unable to deal with this and to explain her behavior since no accusation was ever made openly.

While she was being seen in the Clinic she was working in an office, where she felt that she was not doing work commensurate with her abilities, partly because she had not sufficiently asserted herself in asking for suitable assignments. She had several exacerbations of her skin difficulties in connection with events in the office, as on the occasion when she

found that other employees were given more responsible jobs. About this she said, "I got kind of hot under the collar—it was just another case of 'What are you going to do about it?'—another frustrating situation." About the work which she did, and the behavior of her superior, which was also associated with attacks of dermatitis, she said, "You can't win (the boss), expects more than you can give," and, "The thing that bothers me is her approval." About intellectual abilities in general, she said, "In a sense I feel I'm competing with my father on that score."

There were also difficulties with her husband, some of which were associated with exacerbations of dermatitis. He was not as assertive and ambitious in business as she thought he ought to be, and did not respond well to her attempts to make him increase his efforts. He also failed to help with the housework as much as she would have liked, and she felt that "doing housework just puts me on a level with all those other girls I don't like."

Another aspect of her relations with him was that she "could not keep up with him" in athletics in which they participated, and that "she got no sympathy from him" about her difficulty in this respect.

She made 40 visits to the clinic in a period of a year, and her skin showed marked improvement, although she continued to have intermittent trouble with her hands.

CASE 4.—F. M. was a 28-year-old woman who had itching and some erythema of the hands during part of several successive winters. This had become more severe about two years before her first clinic visit. She had also had occasional episodes of urticaria for the preceding six months.

She had been an only child, who "never gave my mother any trouble," and she and her mother had been "just like sisters." Her father had been "quiet and peace-loving."

Immediately after her marriage she and her husband lived near her parents. Later, however, her father-in-law obtained an apartment for them in the same building in which he and his wife lived. Shortly after this the father-in-law apparently became psychotic, and made various extravagant accusations against the patient, chiefly on the theme of her alleged infidelity to her husband. It was at this time that the first more or less permanent lesions appeared on her hands, and that she also had a transitory episode of dermatitis of the face. Although it was obvious that this was a period of great emotional disturbance, the principal attitudes which she developed were not elucidated.

She complained that her husband had changed since their marriage: "He doesn't want to do a thing around the house." This she found especially trying because of her own perfectionism about housekeeping. She had severe difficulty with itching of her hands while washing the dishes. Her mother-in-law was another source of trouble; once her hands started to itch severely as she was only listening to a conversation in which the mother-in-law was being described. An important feature of the older woman's behavior was that she frequently interrupted the patient in her housework.

She had a 2-year-old baby who also disturbed her. "Now that I have the baby, there are so many things I want to do and can't," and "I just don't like it because she doesn't listen to me." "As soon as I'd get aggravated over it, the first thing I'd do is go for my hands." She had been forced by her husband's family to change the child's name to that of the mother-in-law. She frequently had attacks of itching when upset by the child's behavior.

She made 9 visits to the clinic in a period of three months, during which time her skin showed considerable improvement. She then went away for a vacation with a relative whom she liked, and she wrote that she was almost entirely well. She did not return to the clinic thereafter, and her further course is unknown.

DISCUSSION

It appears from the data presented that there is a specific attitude, susceptible of fairly precise description, which is associated with eczema. It was shown

experimentally that this attitude was associated with cutaneous vasodilatation, involving both the arterioles and the minute vessels, and that at such times the itching and hot skin, which were the chief symptoms of the disease, were aggravated. In short, vasodilatation and increased itchiness seemed to be closely related.

It was found also that the patients in this series seemed to be driven to scratch themselves to an extent somewhat greater than could be accounted for by their itching. The explanation for this seemed to be in large part that they felt a need to punish themselves, and that the scratching was motivated by this need.

It is therefore proposed that the first step in the pathogenesis of the disease is cutaneous vasodilatation, associated with itching, and followed by scratching. It is suggested that the lesions themselves are the result of scratching or otherwise irritating a skin which is the site of vasodilatation, and would not occur otherwise. Probably the amount of trauma necessary to produce a fairly stable lesion varies according to certain characteristics of the skin, among which is the state of the blood vessels.

This vascular pattern of dilatation is, at least within the limits of the experimental techniques employed, exactly the same as that found in cases of urticaria.[10] This similarity is compatible with the opinion expressed by Sulzberger that atopic dermatitis and urticaria are closely related,[20] and the question therefore arises as to what the reasons for the differences in the diseases are. Probably the chief reason for the difference is the excessive scratching by the eczema patients, the reasons for which have been discussed above. The importance of vasodilatation in eczema has been discussed by Gaul and Underwood[5] and is suggested by Williams'[23] experiments in temperature rises in certain skin sites in patients with this disease.

It is to be noted that episodes of urticaria occur fairly frequently in persons with eczema. Five of the patients in the present series had at least one bout of urticaria during the time they were being seen. Furthermore, some of the acute, severe flare-ups of the disease seem to consist of sudden outpourings of fluid into large areas of skin, particularly the face, so that they represent episodes of what amounts to angioneurotic edema or urticaria. In short, really acute exacerbations do not much differ from acute attacks of urticaria.

The physiological explanation for the transudation of fluid is presumably the same as that offered in the case of urticaria[9] and also by Kepecs and associates[14] for atopic dermatitis, namely, that dilatation of the cutaneous vessels increases their permeability.

Eyster, Roth, and Kierland[4] have reported that individuals with atopic dermatitis show more rapid skin cooling than do "normal" persons, and that their skins rewarm more slowly in warm environments. This may indicate that these patients develop vasoconstriction more readily under certain conditions than do other persons. It does not, however, necessarily indicate that vasoconstriction is important in the pathogenesis of the disease, since the authors

do not report evidence that the symptoms and signs were increased by or during the vasoconstriction. In fact, patients with eczema usually state that they feel better while they are in cool environments, or when local vasoconstricting agents such as ice or (in one case) pledgets soaked with epinephrine are applied.

It has been proposed[14] that the transudation of fluid into the skin which is so characteristic of severe dermatitis is to be looked on as expressing the same emotional state as that responsible for weeping; that it is, in short, a kind of weeping into the skin instead of from the lacrimal glands. The experimental findings of these authors, however, indicate that increase in skin fluid occurred with weeping before psychotherapy, but that after therapy weeping occurred without increase in skin fluid.

In the subjects of the present investigation it was found that with really free weeping, associated with attitudes of dejection and hopelessness, there was improvement in the dermatitis, even though of only a few minutes' duration. At such times the skin grew cooler, and itching ceased.

There seems to be no reason, however, to regard weeping as bearing any special relation to dermatitis, although it is probably true that maximal intensity of one of these responses is likely to be associated with diminution in the other. Weeping is one response to stressful situations, and cutaneous vasodilatation is another, and they do not necessarily occur together. Clinical observation shows that the onset of uninhibited weeping is correlated with improvement in many other symptoms, such as asthma or pain from peptic ulcer.

In brief, the dermatitis got worse when the patients felt frustrated, and improved with the onset of other attitudes and their associated bodily changes. Weeping was one of the other possible responses associated with improvement, but so was anxiety, for instance. The question of the specificity of the association of various physiological changes in conjunction with different attitudes is taken up in detail elsewhere.[7]

The correlation found in the present study between an attitude of "frustration"—i.e., of not being able to accomplish what one wishes, without even the ability to think of any way to deal with the obstacles—and exacerbations of the disease, has not been previously reported. The published studies, however, do not indicate that the patient was specifically questioned in order to determine his attitude, as that is defined above.

In general, published protocols do not make possible an evaluation of the precise nature of the patient's feelings, which are usually described by such words as "angry" or "depressed." The word "frustrated" does occur fairly commonly, however, even though a specific relationship of this feeling to the disease is not brought out.

Observation of the patients in this series indicates very strongly that itching occurs at times of vasodilatation. This is probably because itching is the result of low-intensity stimulation of pain endings in the skin, and because vasodilatation (at least of the arterioles) lowers the pain threshold and presumably the itch threshold.[6] This conception of the importance of vascular changes in itching was also arrived at by Gaul and Underwood on the basis of their clinical observations.[5]

Although a consistent *attitude* was found, it is difficult if not impossible to describe a "personality type" for the patients in this series. Kepecs and coworkers[13] found their patients falling into two groups, one "hysterical," the other rigid and compulsive. These two descriptive terms apply to the patients in this series fairly well, except that there was no sharp line of demarcation. In general, however, those whose disease developed in adult life were more likely to have difficulties centering about "work and responsibility" (as Kepecs and collaborators found in their second group) than about guilt and dependence with respect to parenteral figures.

SUMMARY AND CONCLUSIONS

1. Thirty-one patients with eczema were studied to determine the nature of the relation between stressful life situations and exacerbations of the disease, and to investigate the cutaneous processes responsible for the lesions.

2. Exacerbations of eczema occurred in response to situations in which the patient felt that he was prevented from doing as he wished, and was unable even to think of a way to deal with the frustrating circumstances.

3. Experimental study indicated that dilatation of the arterioles and minute vessels of the skin was an important step in the pathogenesis of the disease.

4. This vasodilatation was associated with increased itching.

5. It is suggested that the actual lesions are the result of scratching skin which is the site of vasodilatation. The evidence indicates that persons with eczema, because they are likely to feel guilty and to have urges toward self-punishment rather than toward dealing with their environments, often scratch to an extent out of proportion to the degree of itching.

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EFFECT OF ADENYLATES ON THE LEVEL OF ADENOSINE-TRIPHOSPHATE (ATP) IN THE BLOOD OF NORMAL PERSONS AND OF PATIENTS WITH HODGKIN'S DISEASE

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INTRODUCTION

IN SOME previous experiments[1] we found that the level of nucleotide in the blood of patients with cancer, lymphoma, and various nonmalignant diseases was within the range of values normal to persons in good health except in some instances where there was associated anemia. One of us[2] also reported another series of experiments which showed that 20 mg. of sodium-5-adenylate given intramuscularly to normal individuals brought about a temporary increase in the level of adenosine-triphosphate (ATP) in the blood. Since this increase was not accompanied by an increase in the absolute level of nucleotide, we concluded that it probably represented the conversion into ATP of nucleotide already present. It occurred to us that even though patients with diverse diseases did not show any significant deviation from normal in the total blood nucleotide present, their response to injected muscle adenylic acid might nevertheless be different from the response of healthy persons. In the present paper we report the results of experiments done to ascertain whether or not this is the case.

Data pertaining to normal persons were obtained from experiments done at St. Vincent's Hospital and at Brooklyn College and data on the various disease states from patients at St. Vincent's Hospital. Both zinc-5-adenylate (20 mg. per milliliter) and gelatine preparations of the sodium-5-adenylate (20 mg. per milliliter) were used. It was thought that the gelatine preparation might lengthen the interval of ATP elevation following upon injection.

METHODS

The chemical procedure used for the quantitative measurement of the individual nucleotides has been described by one of us elsewhere.[3] By means of three different enzymes added to properly prepared blood filtrates the adenyl compounds are converted progressively to inosinic acid (IMP) as follows:

$$\text{ATP} \xrightarrow{\text{Hexokinase}} \text{ADP} + \text{Glucose-6-phosphate}$$

$$2\text{ADP} \xrightarrow{\text{Myokinase}} \text{ATP} + \text{AMP}$$

$$\text{AMP} \xrightarrow{\text{Deaminase}} \text{IMP}$$

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Supported in part by grants from the American Cancer Society and the Damon Runyon Memorial Fund.

Received for publication, Dec. 9, 1952.

The extent to which each enzyme converts its specific substrate is measured at 2,650 Å in a Beckman spectrophotometer. From the changes in density registered the amount of each adenyl compound present in the blood can be quantitated. Determination of total purine is made upon filtrate not exposed to enzymes by measuring the density at 2,580 Å and assuming that all the purine present exists as adenine.

Adenylic acid deaminase was prepared according to the method of Kalckar,[4] myokinase according to Colowick and Kalckar,[5] and hexokinase according to Berger and associates.[6] When kept in a subzero freezer these enzymes maintained stability over a long period of time.

In those experiments where sodium-5-adenylate* in aqueous solution was used, blood was withdrawn from the antecubital vein, with heparin as anticoagulant, and deproteinized with perchloric acid. Immediately after the first blood specimen was drawn, 0 time, the adenylate was injected intramuscularly and additional specimens taken thirty, sixty, ninety, and in some cases one hundred twenty minutes afterward. Each specimen was quantitatively analyzed for total purine, adenine present as adenylic acid (AA), adenosine-diphosphate (ADP), and adenosine-triphosphate (ATP). Like quantitation was done on specimens of untreated blood withdrawn at like intervals. As additional controls, analyses were done after injection of 3-adenylate (20 mg. per ml.) and adenosine (20 mg. per ml.). In those experiments where zinc-5-adenylate and the adenylate in gelatine were administered, blood samples were drawn at time intervals up to forty-eight hours, as shown in Table I. Of the 90 blood specimens tested 52 came from normal individuals, 29 from Hodgkin's disease patients, and 9 from a group of persons with various diseases (3 with carcinoma, 3 with cirrhosis of the liver, 1 with rheumatoid arthritis, 1 with congenital heart disease, and 1 with syphilitic heart disease).

TABLE I. EFFECT OF THE INTRAMUSCULAR INJECTION OF 20 MG. OF 5-ADENYLATE, 3-ADENYLATE, ADENOSINE AND 5-ADENYLATE IN GELATINE ON THE LEVEL OF ATP IN THE BLOOD OF ONE NORMAL SUBJECT

DATE		HOURS AFTER INJECTION OF ATP AS ADENINE; MG. %					TOTAL PURINE
		0	½	1	1½	24	INITIAL
9/20/50	Aqueous 5-adenylate	4.0	7.9	6.6	4.8	*	10.0
10/ 9/50	5-adenylate in gelatine	5.9	5.7	6.9	7.7	8.5	9.7
11/ 2/50	Aqueous 3-adenylate	5.1	5.2	5.7	5.5		10.5
11/15/50	Aqueous adenosine	6.1	6.1	6.2	5.8		8.7

RESULTS

Injection of 20 milligrams of the aqueous solution of sodium-5-adenylate into normal individuals produces an increase in the level of ATP in the blood. The maximal increase generally occurs sixty to ninety minutes after injection, after which time the ATP tends to return to its original level. In our original experiment this increase occurred in all the four persons tested; subsequently we found that some normal persons did not show this response. For simplification the percentage of total purine in the form of ATP was calculated initially, and at the time interval when the injected adenylic acid had exerted its maximum influence. The values plotted in Fig. 1 represent, then, the maximum increase in ATP following injection of sodium-5-adenylate, expressed as a percentage of total purine. The first groups of points shown on the figure represent values for patients who received no injection and for patients who

*The adenylates used in these experiments were gifts from the Ernst Bischoff Company, Ivoryton, Conn. These are marketed under the trade name of My-B-Den.

received injections of adenosine or sodium-3-adenylate. We regard these variations as falling within a range reasonably ascribable to experimental error and hence negative. Changes of approximately 10 per cent plus or minus may, therefore, occur when nonspecific substances are injected or when nothing at all is injected.

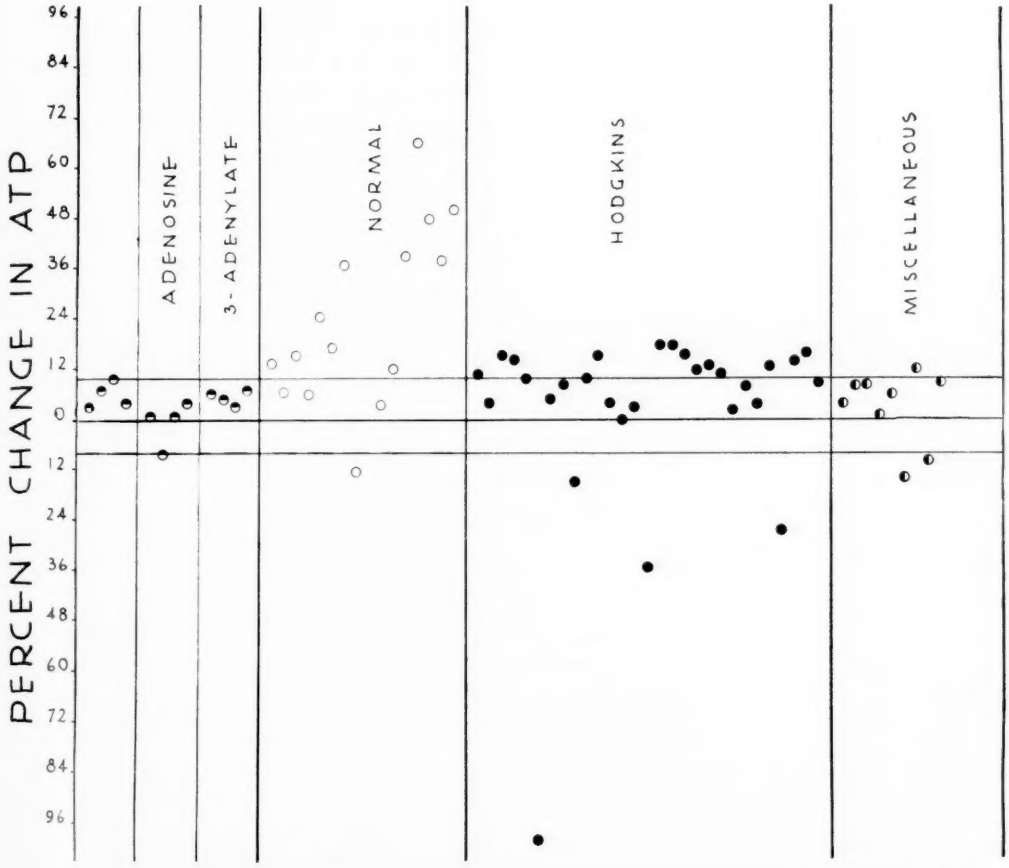

Fig. 1.—Maximum variation (+ or −) in the ATP level of the blood expressed as a per cent change of the ATP initially present, following the intramuscular injection of sodium-5-adenylate in aqueous solution (20 mg.) in normal subjects (○), patients with Hodgkin's disease (●), and patients with miscellaneous diseases (◐) (see text). Each point shown represents a duplicate determination on the blood of a single patient withdrawn at time intervals varying between thirty and one hundred twenty minutes after injection. As controls for the normal group, bloods were withdrawn at similar time intervals following no injection (first group of 4 ◓), when 20 mg of adenosine were given (second group of 4 ◓), and when 20 mg. of sodium-3-adenylate was administered (third group of 4 ◓).

The next set of values resulted from injection of 20 milligrams of sodium-5-adenylate in aqueous solution. These data derive from tests done at Brooklyn College and St. Vincent's Hospital and include earlier, previously reported experiments[2] at Brooklyn College. It should be noted that in this group of determinations only four fell within the normal range; in the remainder the degree of increase varied and in some cases amounted to almost 70 per cent.

In the Hodgkin's disease group, on the other hand, reactions of 12 of 28 patients were within the normal range; 13 were found to have increased ATP, but in no case was the increase so great as was found in some of the normals. In short, where Hodgkin's disease patients responded to the injections of sodium-5-adenylate by and increase in ATP, this increase was on the average much less than that produced in normal persons. When these data were treated statistically, it was found that the average increase produced in normal patients was 25 per cent; in the Hodgkin's group the average increase was 9 per cent. The difference between the means was more than three times their

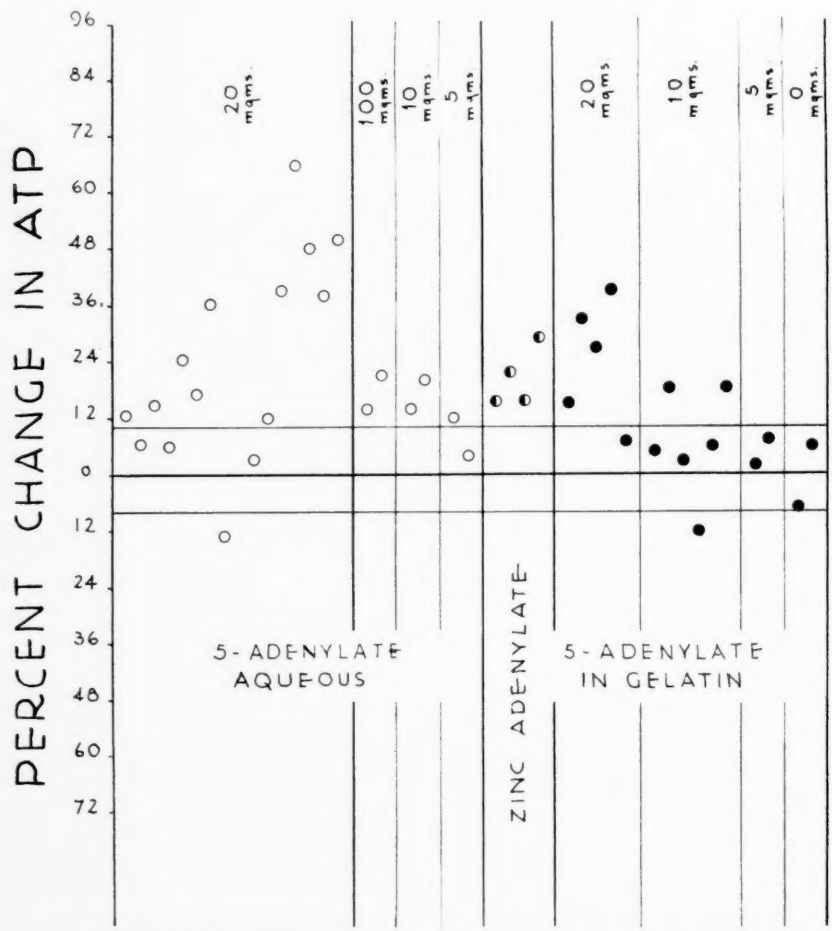

Fig. 2.—Maximum variation (+ or −) in the ATP level of the blood expressed as a per cent change of the ATP initially present, following the intramuscular injection of sodium-5-adenylate in aqueous solution (○) in the various dose levels indicated; zinc-5-adenylate in aqueous suspension (20 mg) ( ); and sodium-5-adenylate in gelatine in the dosages shown (●). Each point represents a duplicate determination on the blood of a single patient. In the case of the sodium-5-adenylate (○) in aqueous solution the maximum change occurred at time intervals varying between thirty and one hundred twenty minutes after injection. In the case of zinc-5-adenylate (◐) it occurred approximately five hours after injection; for the sodium-5-adenylate in gelatine (●) the maximum increase took place twenty-four to thirty hours after injection.

standard error. Reactions of the persons composing the miscellaneous disease group were identical, there being only one whose level of ATP rose slightly above the ranges considered normal.

Some members of the Hodgkin's disease group manifested another interesting response which thus far had been observed only occasionally in the normal group. As shown in Fig. 1, four persons showed a lowered ATP level after injection of sodium-5-adenylate—one person to zero. The significance of this finding is by no means clear, but three of the four Hodgkin's persons died within a short time—nine weeks, one week, and thirty weeks, respectively.

Returning to consideration of the response of normal subjects to the injection of the 5-adenylate, it is of special interest that the smallest dosage of muscle adenylic acid used, 5 milligrams, produced little or no alteration in ATP content of the blood (Fig. 2), but 10 milligrams produced an increase. There appears to be no significant difference in the effect of 20 and 100 milligram injections. In the case of the zinc adenylate, the levels at the end of five hours approximated those following upon 20 milligram doses of the aqueous solution, indicating that the material is absorbed more slowly when present as the zinc salt. As shown in Fig. 2, 20 milligrams of sodium-5-adenylate in gelatin likewise produced a significant change. Here the maximal effect occurs after approximately twenty-four hours and then gradually subsides to normal within the next forty-eight hours. The elevation produced is no more marked than when 20 milligrams of the aqueous solution are given; the only difference is in speed of reaction and the duration of the elevated state. On the other hand, the effects of 5 and 10 milligrams of sodium-5-adenylate in gelatine varied considerably, though in most instances values remained within the normal range. The comparative effects of 20 milligrams of aqueous sodium-5-adenylate, adenosine, sodium-3-adenylate, and 20 milligrams of sodium-5-adenylate in gelatine upon one normal subject are shown in Table I. The gelatine preparation has not yet been tested on any but normal persons.

SUMMARY

Data concerning the effect of intramuscular injection of 5-adenylates and related compounds on the level of ATP in the blood of normal and diseased persons are presented.

Ten milligrams or more in aqueous solution and 20 milligrams in gelatine produced in most healthy subjects a significant elevation above the level established as normal.

Like dosage of the aqueous solution produced no alteration in the level of a large percentage of persons with Hodgkin's disease and, when occurring at all, the rise was much less than that which occurred in members of the normal group. There were even a few persons in this group whose blood showed diminished ATP after dosage.

Findings for a small group of persons with various diseases paralleled those of a group afflicted with Hodgkin's disease.

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THE EFFECTS OF GLUCOSE ON BLOOD PYRUVIC ACID LEVELS IN MILD THIAMINE DEFICIENCY

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INTRODUCTION

THERE is at present no generally accepted laboratory method for detecting early or mild thiamine deficiency. Of the many tests which have been used those which make use of variations in the blood pyruvic acid level have received most attention. The diphosphate of thiamine, co-carboxylase, is required for the decarboxylation of pyruvate, and when the body is deficient in thiamine the normal energy-yielding process of glucose oxidation proceeds only as far as pyruvic acid, which tends to accumulate in the tissues and blood. The pyruvic acid concentration, however, is influenced by many other factors, which affect metabolism.[1] Physical activity and the ingestion of food are particularly important and must be controlled carefully when tests of thiamine deficiency are being performed. These have not always received adequate attention.

In the fasting and resting state very high blood pyruvic acid levels are found in acute thiamine deficiency[2] but not in chronic deficiency. When normal subjects are given glucose by mouth or intravenously the blood pyruvic acid concentration is increased temporarily, but returns to its basal level within three hours. In subjects who are grossly deficient in thiamine the elevation is greater and persists for longer than normal.[3] Tests based on this observation have been found useful for confirming gross clinical deficiency, but not for detecting milder degrees of hypovitaminosis.[4, 5] A more sensitive test has been devised recently[6] in which the concentrations of pyruvic acid, lactic acid, and glucose in the blood are measured after a "double metabolic load," consisting of glucose by mouth and physical exercise (climbing stairs). The relative concentrations of these substances provide an "index of carbohydrate metabolism," which is said to be readily disturbed in even mild thiamine deficiency. This work awaits confirmation.

We wished to investigate biochemically some patients who were thought to exhibit signs of mild or chronic thiamine deficiency.[7] We could not use the "index of carbohydrate metabolism" for two reasons. First, some of the patients had peripheral neuritis and were unable to climb stairs, and second we did not have laboratory facilities for estimating lactic acid as well as pyruvic acid. Moreover, we doubted whether such an elaborate test was necessary and thought that careful study of the curve formed by the blood pyruvic acid levels after the administration of glucose might provide evidence of early or

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Received for publication, Feb. 16, 1953.
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mild thiamine deficiency if the conditions of the test were carefully controlled. As a result of our investigations we found that the earliest detectable change in the curve in thiamine deficiency (at least in our cases) was a prolongation of the time for which the level remained elevated.

EXPERIMENTAL

Subjects.—Thirty-two subjects were investigated, of whom twelve were clinically normal and twenty had undergone gastric resection some time previously. Of the latter group ten had clinical evidence of thiamine deficiency.[7]

Estimation of Pyruvic Acid.—Pyruvic acid was determined by the method of Friedemann and Haugen.[8] The blood was drawn from a vein and immediately pipetted into ice-cold trichloracetic acid to precipitate the proteins. No anticoagulants were used since they affect the pyruvic acid level in various ways.[9] Two identical samples were estimated in each instance, and the mean result was taken. The readings of 100 duplicate samples of blood were submitted to statistical analysis, which showed that the difference between the concentrations of two samples of blood taken from the same subject must be at least 0.13 mg. per 100 ml. if it was to be considered significant at the 0.1 per cent level.

Procedure of Test.—The tests were always performed in the morning, and the subjects were instructed to take nothing to eat or drink after the previous midnight. This fast of nine or ten hours was assumed to be long enough to eliminate the influence of the previous meal on the blood pyruvic acid concentration, since in normal subjects the effects of 100 Gm. of glucose had worn off after three hours and because the initial (fasting) readings obtained in the patients who were deficient in thiamine were the same as those obtained in the normal subjects.

We had no information on the duration or degree of rest which was needed to achieve a basal blood pyruvic acid level. Our results indicated that, after normal activity, rest on a couch for about one hour was necessary to achieve a constant basal level. Therefore, all the subjects on arrival were put to rest on a couch for one hour or longer, after which time a sample of venous blood was taken. They were then given by mouth 100 Gm. of glucose dissolved in 400 ml. of water. Lemon juice was not used for flavoring[4] since citric acid lowers the blood pyruvic acid level.[1] The glucose was usually finished in five minutes, but up to fifteen minutes were allowed. Blood was taken at intervals of one, two, and three hours after finishing the glucose, and pyruvic acid and glucose were estimated in each sample.* Urine was passed after the one-hour specimen had been taken. Throughout the test the subjects remained lying on the couch, except when they sat up to drink the glucose and when they stood up to pass urine. This small amount of exercise did not affect the blood pyruvic acid level.

It has been pointed out[4] that venous occlusion and clenching of the fist, which are often used as aids to venepuncture, may cause false high readings. We found that active clenching and unclenching of the fist for five minutes, with the veins occluded above the elbow, produced an average rise of 0.25 mg. per 100 ml. in the pyruvic acid concentration in blood taken from a forearm vein. For this reason we used a tourniquet as little as possible and never allowed the subjects to clench their fists.

RESULTS

Initial Observations.—Ninety-eight blood pyruvic acid curves were performed on 32 subjects. One subject only had levels which were frankly high (basal, 1.60; 1 hour, 2.00; 2 hours, 2.20; 3 hours, 2.12 mg. per 100 ml.), and at first sight the curves in the rest appeared normal. We noticed, however, that some curves (from both those with intact stomachs and from those who

*The glucose estimation is not part of the test, but was used for research purposes.

had had gastric resections) failed to return to their basal levels in three hours, although the basal and peak levels were normal. We thought that this feature might be the earliest detectable change in thiamine deficiency. There were, however, two other possibilities:

1. The rate of absorption of glucose and the height of the blood glucose peak might affect the form of the pyruvic acid curve, especially since a high proportion of the gastrectomized subjects had glucose tolerance curves of the "lag storage" type.[10] We could, however, find no consistent relationship between the 2 curves. It was of interest that 2 patients had marked hypoglycemic symptoms three hours after taking glucose, and that their pyruvic acid levels rose steeply at this time. In one, who actually lost consciousness, the level reached 3.10 mg. per 100 ml. These curves have been excluded from the analysis.

2. Some of the patients became rather restless after lying down for four hours, and we thought that secretion of epinephrine at this time might, by stimulating glycogenolysis, be causing a terminal rise in the blood pyruvic acid level,[1] particularly since in some patients the level was higher at the third than the second hour. For this reason we measured the heart rate and blood pressure immediately before taking each specimen of blood in the subsequent tests, but in no case was there a significant increase in either at the time of taking the three-hour specimen. We therefore concluded that there was no undue secretion of epinephrine.

Effect of Thiamine Therapy on Pyruvic Acid Curves.—It remained to investigate the effect of thiamine therapy on these blood pyruvic acid curves. Twenty subjects were observed for periods varying from one to twelve months without treatment, and 18 subjects were observed both before and after treatment. Treatment consisted in giving tablets of thiamine hydrochloride by mouth in either "light" (up to 10 mg. per day) or "heavy" (usually 25 mg. three times a day) dosage, and in most cases light dosage was given first. Treatment was always given for at least four weeks and was continued up to the time of performing the next test. The results are shown in the tables. Since no evidence was found to suggest that those with intact stomachs behaved differently from those who had had gastric resections (Table I), the results from these two groups have been combined (Table II). It is clear (Table II) that thiamine therapy had no effect on the basal level, but that it did lower the peak and also the level to which the pyruvic acid fell after the peak. This last effect is seen most clearly by measuring the difference between the basal level and the two- or three-hour level (whichever is lower). Before treatment this difference was on the average + 0.231 mg. per 100 ml. and after treatment approximately zero (–0.031 mg. per 100 ml.). Moreover, once it had been reduced to zero it tended to remain so provided treatment was continued. Conversely, the difference inexplicably tended to increase in subjects who were not treated (Table I). We therefore felt justified in assuming that a curve which failed to return to the basal level within three hours represented a state of thiamine deficiency.

TABLE I. CHANGES IN PYRUVIC ACID CURVES. (ALL READINGS IN MG. OF PYRUVIC ACID PER 100 ML. OF BLOOD)

A. Without Treatment.
(Results of 2 tests at intervals of 1 month to 1 year.)

PYRUVIC ACID LEVELS	SUBJECTS WITH INTACT STOMACHS (MEAN LEVELS IN 11 SUBJECTS)				GASTRECTOMIZED SUBJECTS (MEAN LEVELS IN 9 SUBJECTS)			
	TEST NO.		CHANGE	SIGNIFICANCE LEVEL OF CHANGE	TEST NO.		CHANGE	SIGNIFICANCE LEVEL OF CHANGE
	1	2			1	2		
Basal	0.9464	0.9354	−0.0110	N.S.	0.7433	0.9100	0.1667	N.S.
Peak	1.3409	1.4091	0.0682	N.S.	1.5278	1.7411	0.2133	N.S.
Lowest after peak	0.8136	0.9736	0.1600	N.S.	0.8733	1.0933	0.2200	0.05
Difference (lowest-minus-basal)	−0.1327	0.0382	0.1709	0.02	0.1300	0.1733	0.0333	N.S.

B. With Thiamine Treatment.
(Results of last test before, and last test after, treatment.)

PYRUVIC ACID LEVELS	SUBJECTS WITH INTACT STOMACHS (MEAN LEVELS IN 3 SUBJECTS)				GASTRECTOMIZED SUBJECTS (MEAN LEVELS IN 15 SUBJECTS)			
	BEFORE	AFTER	CHANGE	SIGNIFICANCE LEVEL OF CHANGE	BEFORE	AFTER	CHANGE	SIGNIFICANCE LEVEL OF CHANGE
Basal	0.8533	0.9266	0.0733	N.S.	0.8707	0.9953	0.1246	N.S.
Peak	1.5733	1.2800	−0.2933	N.S.	1.8567	1.6846	−0.1721	N.S.
Lowest after peak	1.0733	0.7833	−0.2900	N.S.	1.1033	0.9887	−0.1146	N.S.
Difference (lowest-minus-basal)	0.2200	−0.1500	−0.3700	0.05	0.2327	−0.0067	−0.2394	0.001

In determining whether the mean changes were significantly different from zero the following estimations of the variance of individual changes have been used for calculating standard error:

Basal	−0.0801
Peak	−0.1761
Lowest	−0.0834
Difference	−0.0439

TABLE II. EFFECTS OF THIAMINE ON BLOOD PYRUVIC ACID LEVELS

PYRUVIC ACID LEVEL	MEAN EFFECT OF TREATMENT AND STANDARD ERROR (MG. PER 100 ML. OF BLOOD)		"t" (34 D.F.)	LEVEL OF SIGNIFICANCE
Basal	−0.0377	±0.1002	0.376	N.S.
Peak	−0.3783	±0.1508	2.508	0.02
Lowest after peak	−0.3687	±0.1022	3.608	0.01
Difference (lowest-minus-basal)	−0.3519	±0.0741	4.746	0.001

The data have been analyzed according to the method given by Snedecor,[11] and no evidence has been found to suggest that the controls and the gastrectomized patients responded differently to treatment. The estimates from the two groups have therefore been combined. The effects of treatment have been compared with the effects of giving no treatment.

Classification of Pyruvic Acid Curves.—With this assumption we re-examined all our curves and divided them into two groups:—

A. (Presumed normal) Those in which the difference (lowest after peak minus basal) was not significant (i.e., less than 0.13 mg. per 100 ml.), and

B. (Presumed deficient) Those in which the difference *was* significant (i.e., 0.13 mg. per 100 ml. or more).

A few of the Group A curves, however, had basal levels which were unusually high (up to 1.87 mg. per 100 ml.) and could not strictly be regarded as normal. We therefore made arbitrary distinction between those in which the basal level was less than 1.20 mg. per 100 ml. (Type 1) and those in which it was higher (Type 2). A similar distinction was made in Group B (Types 3 and 4). We now had 4 types of curve with the following features (see Fig. 1):

1. Basal level 0.50 to 1.19 mg. per 100 ml.; Peak 1.00 to 2.20 mg. per 100 ml. at one hour; Return to basal level within three hours (i.e., at the two- or three-hour reading, whichever is lower).

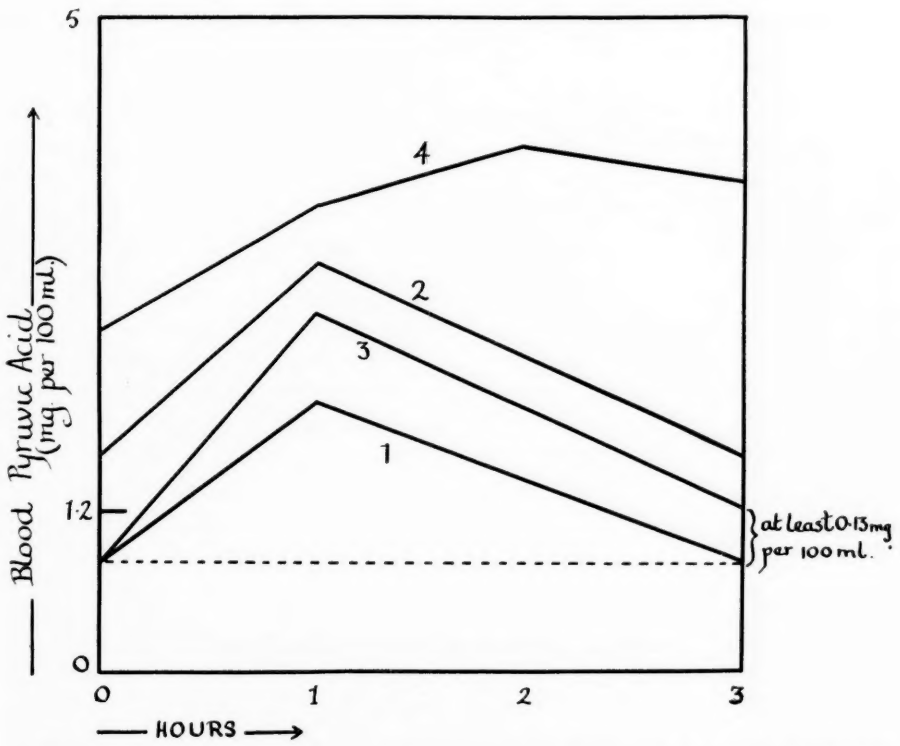

Fig. 1.—Showing the main types of blood pyruvic acid curve obtained after giving 100 Gm. of glucose by mouth. *1* is normal, *2* is difficult to interpret, but is probably normal, *3* and *4* indicate thiamine deficiency. For details see text.

2. Basal level 1.20 mg. per 100 ml. or higher; Peak 1.50 to 2.60 mg. per 100 ml. at one hour; Return to basal level within three hours.
3. Basal level 0.50* to 1.19 mg. per 100 ml.; Peak 1.00 to 3.00 mg. per 100 ml. at one hour. Lowest level after peak significantly higher than basal level.
4. Basal level 1.20 mg. per 100 ml. or higher; lowest level after peak significantly higher than basal level.

*An isolated reading of 0.22 mg. per 100 ml. was obtained.

TABLE III. TYPES OF BLOOD PYRUVIC ACID CURVE OBTAINED IN ALL SUBJECTS BEFORE AND AFTER TREATMENT WITH THIAMINE

SUBJECT*	TYPE OF BLOOD PYRUVIC ACID CURVE							
	BEFORE TREATMENT†		AFTER TREATMENT					
			LIGHT		HEAVY			
Normal Curves (1 and 2)								
Clinically normal								
M. A.	1	2						
G. B.	1	1						
R. B.‡	1	1						
D. C.	1	1						
Z. D.	1	1						
B. D.	1	1						
R. E.‡	1	1						
N. F.‡	1	1						
A. F.	1	1						
J. F.‡	2				2	1		
B. G.	1		1		1			
F. G.‡	1		1					
W. L.	1	1						
C. L.	1	1						
B. W.	1	1						
Clinically deficient								
T. J.‡	1		2		1	2		
J. R.‡	1		1					
Deficiency Curves (3 and 4)								
Clinically normal								
P. D.‡	(3)	1						
J. D.‡	3		1	(3)	1	1		
H. E.‡	3		1	1				
W. H.‡	3		1	1§				
M. L.	3	3	3		1	1		
T. M.‡ (2)	1	(3)						
K. W.	2	3			1	1		
Clinically deficient								
T. A.‡ (4)	3		3		1	1		
S. B.‡	3		3	1	1			
R. J.‡	4	4	1		1	1		
C. K.‡ (8)	3		(1)	3	3	1‖	1	1
W. P.‡	3	3	1	1				
G. R.‡ (3)	3	3			2			
I. S.‡	3		1					
H. S.‡	3	3			3	3	1‖	3‖

*The figures after the initials of some of the subjects in this column refer to the numbers under which they were described in a previous paper.[7]

†Where two figures are shown in this column there was an interval of from one to twelve months between the two tests.

‡Subjects who had undergone gastric resection.

§A Type 3 curve occurred again six months after stopping therapy.

‖Co-carboxylase given before these tests.

By accepted standards[3, 4] a Type 1 curve is normal and a Type 4 curve suggests thiamine deficiency. We have presented the evidence for supposing that Type 3 represents deficiency also. We are not sure of the significance of Type 2, but are supposing for the moment that it is normal.

Table III shows the types of curve which were obtained before and after treatment with thiamine in all the subjects. In general, when no treatment was given the type of curve did not alter. A deficiency curve became normal in some cases after light treatment and in all but two cases after heavy treatment. In these two (C. K. and H. S.) the effect of co-carboxylase (100 mg. intravenously daily for varying short periods) was observed. In both it appears to have been biochemically effective, but in one (H. S.) its effect was not maintained.

The four curves in brackets (J. D., C. K., T. M., and P. D.) are inexplicably inconsistent with the general scheme, although T. M., who had undergone gastric resection, may well have developed deficiency during the interval between his two tests.

Correlation Between Pyruvic Acid Curves and Clinical State.—A clinical diagnosis of thiamine deficiency was made when established peripheral neuritis was present, for which no other cause could be found, or when symptoms such as paresthesias or fatigue were relieved following treatment (either light or heavy) with thiamine.

Before treatment 17 subjects had normal (Types 1 and 2) curves (Table III), and two of these had clinical evidence of deficiency. Fifteen had abnormal (Types 3 and 4) curves at some time, and eight of these had clinical evidence of deficiency. That is to say that the biochemical test confirmed the clinical diagnosis in eight of ten cases, failed to confirm it in two, and revealed evidence of deficiency in seven of twenty-two subjects in whom it was not suspected.

The clinical response to treatment usually coincided with the biochemical response, although one sometimes preceded the other.

DISCUSSION

So far as we know it has not previously been shown that a blood pyruvic acid curve which has its basal and peak levels within the normal range, but which fails to return to the basal level within three hours, is abnormal and can be altered by thiamine therapy. Indeed, most workers have continued their observations for only one and one-half to two hours after giving glucose.[5, 12] The test is easier to perform than one which involves the estimation of lactic acid and glucose as well as pyruvic acid, and it *does* appear to be of value in recognizing mild and chronic cases of thiamine deficiency. It certainly deserves further trial.

It is true that the results were sometimes equivocal, but, considering the number of factors which may influence the blood pyruvic acid level, they were remarkably consistent. We believe that careful control of physical activity, of fasting, and of the details of blood collection were largely responsible for this. Further, the difficulties which often arise from trying to apply a statistical phenomenon to an individual problem (e.g., in relying on an isolated blood pyruvic acid estimation for evidence of thiamine deficiency) have been largely overcome by measuring the difference between two observations (the basal level and the lowest level after the peak) in the same subject.

It is difficult to understand why such large doses of thiamine are sometimes needed to alter the pyruvic acid curve and to relieve symptoms. This is probably related to the cause of the vitamin deficiency. Possibly some subjects are unable to absorb thiamine normally. Those whose curves remain abnormal in spite of massive therapy may either have other deficiencies or fail to utilize thiamine in the body. Our results with co-carboxylase are interesting but inconclusive, and such cases require further study.

SUMMARY AND CONCLUSIONS

An analysis is given of 98 blood pyruvic acid level/time curves, following the administration of glucose, in 32 subjects. A test has been devised which provides evidence of early or mild thiamine deficiency. The blood pyruvic acid level is measured in fasting subjects after one hour's rest and again at one, two, and three hours after taking 100 Gm. of glucose by mouth. The subjects remain at rest throughout the test. Thiamine deficiency can be presumed if the blood pyruvic acid concentration fails to return to its basal level after three hours, even when the basal and peak levels are normal. In most cases treatment with thiamine restores the curve to normal. The test has provided confirmation of a clinical diagnosis of thiamine deficiency (based on the response to specific therapy) in eight of ten cases and has given evidence of deficiency in cases where it had not been suspected.

Acknowledgments.—We wish to thank Professor Charles Wells and Dr. Robert Hughes for their constant help and encouragement; Dr. Fred. Wrigley of Roche Products Ltd., for his helpful advice and for generous supplies of vitamins and co-carboxylase; Mrs. Zoe Dreyer, M.D., Budapest, for the glucose estimations. Mr. Kenneth Gordon, B.Sc., for his invaluable help with the statistical analyses; and finally our long-suffering subjects, particularly Miss Barbara Duckworth and Miss Maureen Litchfield, both of whom acted as experimental subjects many times.

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CLINICAL EXPERIENCE WITH A NEW ORAL MERCURIAL DIURETIC, 3-CHLOROMERCURI-2-METHOXYPROPYLUREA (1347 EX)*

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THE role of organic mercurials in the treatment of congestive heart failure has been well established. It is generally accepted that the parenteral route is the most effective mode of administration for a rapid and immediate diuresis. For maintenance purposes, however, oral therapy would be superior provided an effective and nontoxic preparation were available. The purpose of the study was to investigate clinically such a new oral mercurial diuretic, 3-chloromercuri-2-methoxypropylurea (1347 EX) (Neohydrin).*

Neohydrin is a product resulting from the combination of mercuric acetate with allylurea in methyl alcohol.[1] The preparation is made in the form of an uncoated tablet containing 18.35 mg. of the compound equivalent to 10 mg. of mercury.

MATERIAL AND METHODS

Fifteen ambulatory cardiac patients were studied. The specific diagnoses, duration of therapy, dosage of medication, and therapeutic results are summarized in Table I. All patients except one (D. M.) had been observed for a control period of at least one year. During this time (with the exception of E. T.) each patient received mercurial diuretics parenterally for the management of congestive heart failure. The amount of parenteral diuretic necessary for maintenance of a stable state, as far as evidence of fluid retention was concerned, was determined in each patient (Table I). During the period of investigation an attempt was made to replace completely the parenteral mecurial with the oral preparation and still maintain the patients free from signs of congestive failure. During this study all patients, except one, were digitalized, and each observed moderate salt restriction. The clinical effects of the oral preparation were compared with these obtained during the period of parenteral therapy. An attempt was made to see each patient weekly, and particular attention was directed toward the manifestations of fluid retention (weight gain, pedal edema, hepatomegaly, dyspnea, orthopnea, and râles). Untoward effects of the drug were specifically sought for at each visit. Placebos were not used. Laboratory studies included a hemogram, serum urea nitrogen, and urinalysis; electrocardiograms were obtained when clinically indicated.

OBSERVATIONS

The response of each patient to the oral mercurial was classified as "good" if the parenteral mercurial was completely replaced or required infrequently. Ten of the fifteen patients were classified in this group. A "fair" response consisted of partial replacement by the oral preparation or an uncertain

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Aided by grants from the Lakeside Laboratories and the Michael Reese Research Foundation.

Received for publication, Mar. 6, 1953.

*This was supplied by Dr. H. L. Daiell of Lakeside Laboratories.

TABLE I. SUMMARY OF CLINICAL DATA

PATIENT	AGE (YEARS) AND SEX	DIAGNOSIS	PARENTERAL MERCURIAL (MERCUHYDRIN OR THIOMERIN) DURING CONTROL PERIOD		DOSAGE OF DRUG (TAB./DAY)		RESPONSE	COMMENTS
			AMOUNT	DURATION OF THERAPY	REPLACEMENT	MAINTENANCE		
1. S. M.	75 F	ASHD	1 c.c./10-14 days	8½ mo.	3	2	Good	Complete replacement of parenteral drug.
2. F. R.	84 F	ASHD	1-2 c.c./week	7½ mo.	3	2	Good	Complete replacement of parenteral drug.
3. W. L.	50 M	ASHD	2 c.c./3 weeks	11½ mo.	1	1	Good	Complete replacement of parenteral drug.
4. D. M.	51 M	RHD	2 c.c./2 weeks	7 mo.	3	3	Good	Complete replacement of parenteral drug.
5. L. M.	65 F	HCVD	2 c.c./week	8½ mo.	3	1	Fair	Complete replacement, but suddenly; went into failure. Acute myocardial infarct?
6. E. D.	45 F	HCVD	2 c.c./2 weeks	10 mo.	3	2	Good	Complete replacement of parenteral drug.
7. L. K.	62 F	ASHD	2 c.c./week	13 mo.	3	1	Good	Complete replacement of parenteral drug.
8. C. L.	64 F	ASHD	1 c.c. intermittently	7 mo.	3	1	Good	Complete replacement of parenteral drug.
9. D. Z.	85 M	ASHD	1 c.c./week	3 mo.	3	2	Fair	Did well on oral preparations, but classified as fair because of poor follow-up.
10. H. B.	75 M	HCVD	2 c.c./week	10 mo.	3	3	Good	Complete replacement of parenteral drug.
11. S. G.	78 M	ASHD	1 c.c./5-7 days	6½ mo.	2	2	Good	Complete replacement for 4 mo. then needed parenteral drug one time.
12. E. T.	64 F	HCVD	None	7 mo.	2	2	Fair	Classified as fair, because response was "good," but there was nothing to replace.
13. D. L.	56 F	RHD	2 c.c./7-10 days	6 mo.	—	3	Fair	Requires 2 cc parenteral every 3 weeks.
14. B. B.	69 M	ASHD	2 c.c. 3 ×/week	4½ mo.	3	3	Good	Complete replacement of parenteral drug.
15. V. F.	42 F	SHD RHD	2 c.c./week	4 mo.	3	3	Good	Excellent response, but still requires 1 cc parenteral every 4-6 weeks.

ASHD = Arteriosclerotic heart disease.
SHD = Syphilitic heart disease.
RHD = Rheumatic heart disease.
HCVD = Hypertensive cardiovascular disease.
TAB. = Tablets (18.35 mg. = 10 mg. of mercury).

"good" response. Five patients fell into this category. In none of the patients included in this study did we see a "poor" result, that is, total ineffectiveness of the drug. Several of the patients were kept for a time without the oral or parenteral mercurial. In each instance evidence of fluid retention soon ensued, but disappeared upon reinstitution of mercurial therapy.

The dosage of the oral preparation was found to average three tablets daily for replacement during the first week or two of therapy and one to three tablets daily for maintenance (Table I). Although the tablets were usually taken with meals there were no complaints of intolerance if ingested on an empty stomach.

Untoward effects including stomatitis, severe diarrhea, cramps, or vomiting, were not encountered in any of the fifteen patients reported in this study.* All patients were cautioned to exercise good oral hygiene.

Albuminuria was noted in five of the ten patients tested, and a slightly elevated serum urea nitrogen was observed in three of nine patients examined. Neither the albuminuria nor the azotemia could be attributed to the oral mercurial. In the nine patients on whom electrocardiographic and hematologic examinations were performed there were no changes that could be ascribed to the oral mercurial preparation.

All patients preferred the oral to the parenteral route because the discomfort associated with the injection was avoided. Freedom from the distressing symptoms resulting from profuse diuresis and its attendant nocturia was also noted.

DISCUSSION

For many years organic mercurials have been used in the treatment of congestive heart failure. Intravenous, intramuscular, and subcutaneous preparations have been the preferred routes of administration. However, certain disadvantages and limitations to parenteral therapy exist. Frequently, complications have arisen from too rapid a diuresis, e.g., attacks of gout[2] and tetany[3] have been precipitated as well as the "low salt syndrome." Cerebral thromboses[4] also have been blamed on too vigorous therapy. Similarly overdigitalization has been commonly observed following the sudden elimination of large quantities of fluid from the body. Pain at the site of injection is frequently experienced with parenteral therapy. Administration of the mercurial parenterally requires the service either of a physician or of a nurse, thus adding to the economic burden to the patient or community.

Since ease of administration and a steady diuretic effect would be expected of an oral mercurial, many attempts have been made to find an effective and nontoxic oral preparation. Most of the oral mercurials tried heretofore have been poorly tolerated, giving rise to stomatitis, gingivitis, abdominal cramps, vomiting, or diarrhea. The effectiveness of the oral preparations generally has been below that of the parenteral drugs.

*In two other patients not included in this study cramps and diarrhea did occur. One was treated with the oral mercurial for premenstrual tension, and another was given the drug for nonspecific lymphedema of the lower extremities. The diarrhea and cramps disappeared when the dosage was reduced. An erythematous rash was seen in one patient who was given the oral preparation. This rash disappeared when the oral drug was withheld, but reappeared when a parenteral mercurial was administered.

Handley and associates[5] have shown in the dog that 3-chloromercuri-2-methoxypropylurea given intravenously had three to four times the diuretic potency of meralluride (mercuhydrin). Chronic toxicity studies revealed no essential difference between the new compound and meralluride if the greater diuretic potency of the former was considered and the dosage adjusted accordingly. The acute cardiac toxicity of 3-chloromercuri-2-methoxypropylurea appeared to be less than that of meralluride. Griener and co-workers[6] have bio-assayed the new oral mercurial and have demonstrated that this compound possesses three-fourths the diuretic potency of the standard (mercuhydrin by intramuscular administration). All other oral preparations so far tested have come no nearer than one-fourth the diuretic potency of the standard.

Our experience with 3-chloromercuri-2-methoxypropylurea orally suggests that it has promise as an adequate oral mercurial diuretic without undue toxic effects. It is worthy of further trial.

SUMMARY

A new oral mercurial diuretic, 3-chloromercuri-2-methoxypropylurea (1347 EX) was administered to fifteen ambulatory cardiac patients as part of the treatment of congestive heart failure.

Complete replacement of the parenteral mercurial was brought about in ten patients and partial replacement was possible in the other five.

The drug as a rule was well tolerated and no serious toxic signs were encountered.

From our preliminary observations it appears that the oral preparation under investigation is safe and effective if administered properly.

We are indebted to Dr. L. N. Katz for his suggestions in the conduct of this study and in the preparation of this report.

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A STUDY OF BACTERIA IMPLICATED IN TRANSFUSION REACTIONS AND OF BACTERIA ISOLATED FROM BLOOD PRODUCTS

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THE purpose of this paper is to focus attention on the characteristics of bacteria that have gained access to blood and blood products. The study of the bacteria from blood was prompted by the recurring reports of severe and fatal reactions following administration of bacterially contaminated blood. Bacterial contaminants from blood products are included to give a better understanding of the total problem of bacterial contamination in the handling of blood and blood products.

Eighteen severe or fatal reactions have come to the attention of the Laboratory of Biologics Control. This number could be considered as a fraction of the total number that are occurring, since they were heard about incidentally. Only two of the eighteen have been mentioned before in the literature.[1] Besides their report of these two, Borden and Hall collected from the literature 15 others; 8 of the 17 were fatal; plasma as well as blood was involved. Five additional blood transfusion reactions ascribed to bacterial contaminants have been found recorded in the literature. Three, caused by bacteria of the coli-aerogenes group, were fatal.[2-4] The other two, nonfatal, were due, respectively, to a diphtheroid and a suspected enterococcus.[5] An enterococcus had been isolated four days previously, but no culture was made at the time of transfusion. Borden and Hall[1] consider that febrile reactions due to bacterial contamination occur more frequently than is realized. They cite instances to show that there has existed a lack of consideration of bacteria as one of the causes.

Besides the alarming reactions there are those of less severity ranging down to the very mild ones. The true incidence of all febrile responses following blood or blood product transfusions is difficult to determine. The literature has been reviewed.[3, 6, 7] There has been a decrease since the very early reports that every transfusion was followed by a reaction to later ones that indicate less than 10 per cent. Scott[6] compiled the data on 25,459 transfusions reported from the Mayo Clinic for the seven years, 1937 to 1943, and found the incidence to be 9.04 per cent. For the three years, 1949 to 1951, however, the rate was down to 5.3 per cent for 22,230 transfusions.[8-10] The over-all fatality rate can be estimated only. Moore[11] estimated it at 0.14 per cent, while Moore[12] considered that the published reports of 0.01 to 0.03 per cent are probably minimum.

Presented in part before the Fifth Annual Meeting of the American Association of Blood Banks, Milwaukee, Wis., Oct. 11, 1952.

From the Laboratory of Biologics Control, National Microbiological Institute, National Institutes of Health, Public Health Service, Federal Security Agnecy.

Received for publication, Feb. 27, 1953.

It has been claimed that the majority of the reactions are due to nonspecific agents[7, 13] and that the contamination of the blood or apparatus by pyrogenic agents is the major cause.[14, 15] What portion is due to viable bacteria is not known. In the earlier reports the bacterial contamination rates ranged from 5 to 25 per cent.[2, 16, 17] In the most recent one, Braude and associates[5] reported a contamination rate of 2.24 per cent for 1697 bloods that were cultured after twenty-four hours' storage at 4° C. but the rate was 4.5 per cent for 200 bloods that were cultured during transfusion. They used 37° C. for incubation as has been used generally in blood studies, although it has been stated that some contaminants do not grow at this temperature.[3, 18, 19, 20]

In this paper will be presented the results of a study of the bacteria isolated from some of the bloods implicated in the transfusion reactions reported to us, and from plasma and albumin. It will include the relative distribution of the different kinds of bacteria among the products, the classification of the bacteria as far as was possible for us to determine, and the growth temperature range of representative bacteria.

EXPERIMENTAL RESULTS

Source and Broad Grouping of the Cultures.—In the early part of the plasma and albumin programs of World War II, eight processing laboratories sent to us a number of cultures that had been isolated at some stage of the processing. The pyrogenicity of representative cultures was studied by Probey and Pittman[21] in order to obtain information on the maximum number of bacteria that could be present in plasma and not leave it pyrogenic after filtration. Some of the cultures were also used in studies of the sterility test medium[19] and of preservatives for plasma.[22] The cultures from blood came directly or indirectly from the laboratory that made the primary isolation. In our collection, the number assigned to a contaminant carrys a prefix PC; it is omitted in this paper for brevity.

Ninety-eight cultures from blood and blood products and eleven from vaccines and antigens have been studied. The distribution of the 109 cultures among four groupings of the products is given in Fig. 1. The bacteria fall into five morphological groups. The number in each group and the relative incidence of the number in a group to the total number from a product is given by percentage. The cultures from plasma and albumin were isolated when the sterility test was being incubated at 37° C.

From plasma both the kinds of bacteria and their relative distribution are in fair agreement with the findings reported for blood when the isolations were made by incubating the test for sterility at 37° C.[5, 23] Gram-positive bacteria have usually predominated with micrococci occurring in the greatest number, although Novak[16] reported sporeformers as being most frequent. In contrast, Johnson and Meleney[18] recovered a larger number of gram-negative bacteria from plasma. These were twice as numerous as the micrococci, but the relative incidence of streptococci, sporeformers, and diphtheroids was similar to that among our cultures from plasma. Their procedure differed

from that used by the others. The plasma was held at room temperature for a week before the sterility test which was performed at both 37° C. and room temperature. It is of particular interest that of the 47 contaminants they recovered, twenty were of the genus *Pseudomonas*. Some of the latter did not grow at 37° C.

From albumin, the micrococci comprised 70 per cent of the cultures. No streptococci were present. The relative numbers of sporeformers, diphtheroids,

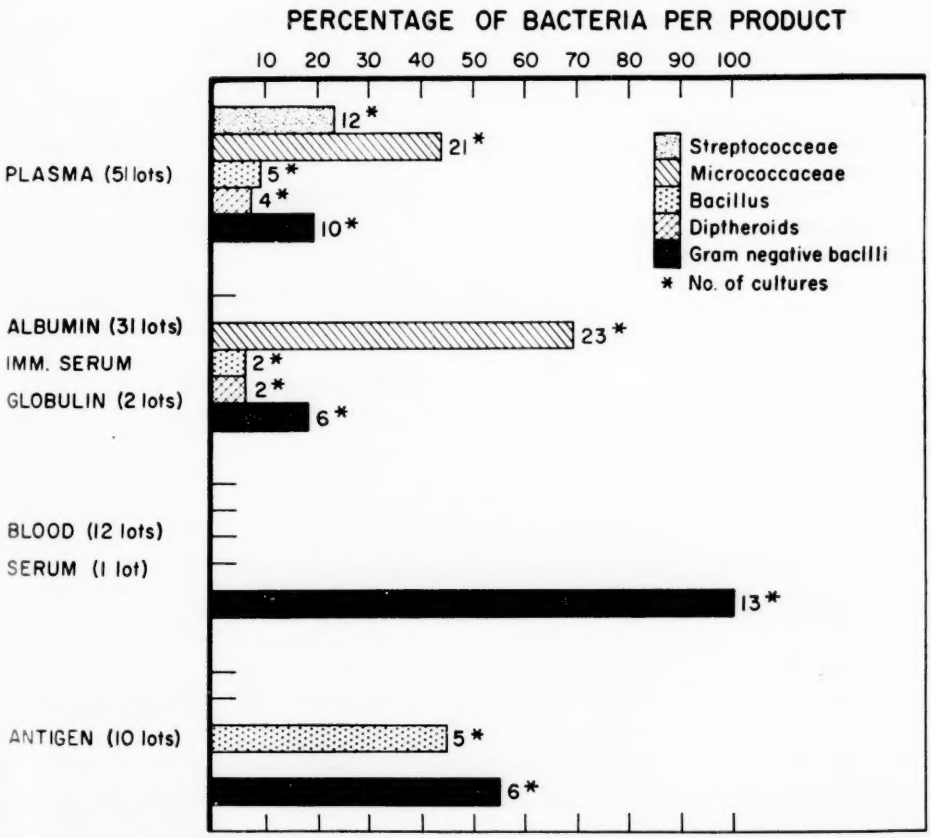

Fig. 1.—The distribution of morphological groups of bacteria isolated from blood, blood products, and antigens and sent to the Laboratory of Biologics Control.

and gram-negative bacilli are about the same as from plasma. The cultures from blood and serum, all gram-negative bacilli, compose a select group, as each had been isolated from a product that had induced a transfusion reaction or from a related blood that had been recalled. From antigens and vaccines, only sporeformers and gram-negative bacilli were obtained. All of the latter came from vaccines harvested from extraembryonic fluid of fowl eggs.

The Bacteria Implicated in Transfusion Reactions.—In Table I are given both the outcome of the reactions, that were called to the attention of the

TABLE I. BACTERIA IMPLICATED IN TRANSFUSION REACTIONS REPORTED TO THE LABORATORY OF BIOLOGICS CONTROL

PROCESSOR OR BANK	DATE	PRODUCT	REACTIONS NO.	REACTIONS OUTCOME	BACTERIA IMPLICATED
A	1944	Serum	1	Recovered	*Pseudomonas*
B	1948	Blood	1	Fatal	*Pseudomonas*, "*Achromobacter*"*
		Blood	1	Fatal	*Paracolobactrum aerogenoides**
C	1951	Blood	1	Fatal	"*Alcaligenes faecalis* and *E. coli*"†
D	1951	Blood	2	Fatal	"*Paracolobactrum intermedium*"‡
	1952	Blood	1	Fatal	*P. aerogenoides*
		Blood	1	Fatal	"Hemolytic *coli*"
E	1951	Blood	2	Fatal	
		Blood	1	Died after 7 days	*Pseudomonas* and/or *Escherichia freundii*§
		Blood	5	Recovered	
F	1952	Red cells	1	Fatal	*Pseudomonas*
G	1952	Red cells	1	Recovered	"Gram-negative bacilli in smear—no growth at 37° C."‖

Quotation marks indicate the identification reported to us.

*Reported by Borden and Hall.[1]

†Identification made by Dr. J. Douglas Reid, Richmond, Va.

‡Probably *P. aerogenoides* as acetylmethycarbinol was produced. The same bacterium was isolated from three recalled bloods drawn on the same day.

§All bloods involved were not cultured, but eight cultures were received that were from implicated or recalled bloods drawn on the same day.

‖Failure to grow at 37° C. suggests that this culture was of *Pseudomonas*.

Laboratory of Biologics Control and the bacteria that were isolated from the transfusion fluids. On the basis of the case histories and the bacteriologic findings, the etiological role of bacteria in these 18 reactions seems beyond question. In certain instances, the bacteria were observed in direct smear of the transfusion fluid as well as being recovered in culture, and in others, the bacteria were isolated also from the recipient. In two bank episodes, bloods drawn on the same day induced reactions in two or more patients; a bacterium in common was isolated from the residues of the bloods administered and also from companion bloods recalled to the bank.

The bacteria given in quotation marks in Table I were studied by others and those without the marks by us. The majority were either of the genus *Pseudomonas* or the coli-aerogenes group. *Alcaligenes faecalis* was reported to be isolated from the Bank C blood. There is a disagreement on the identification of the bacterium from the first blood of Bank B. Dr. Hall kindly sent to us the cultural characteristics of this bacterium from Bank B that they observed. Their culture, which was lost, and the one from the same source that we studied gave the same biochemical reactions. The two cultures differed in that the former produced no pigment and grew slowly at 37° C., while the latter produced soluble yellow-green pigment and did not grow at 37° C. (see No. 113 in Table IV). The latter was lophotrichous, while the location of flagella on the former was not reported. Dr. Hall also stated that two cultures, sent at different times, were studied at the Army Medical Department Research and Graduate School. The first received was reported to be *Pseudomonas* and the second *Achromobacter*. Possible explanations for the discrepancy are that (1) one

TABLE II. THE GRAM-NEGATIVE BACTERIA FROM BLOOD PRODUCTS, BLOOD, AND ANTIGENS

BACTERIA	PLASMA	NUMBER OF CULTURES FROM				
		ALBUMIN	SERUM	BLOOD	ANTIGEN	TOTALS
*Pseudomonas**	1	1	1	7	3	13
E. freundii	1	1		3	2	7
P. aerogenoides†	4			2		6
Aerobacter cloacae	2					2
A. faecalis		2				2
Unidentified	2	2			1	5
Totals	10	6	1	12	6	35

*Several species are represented, see Table III.
†One strain, No. 58, may be *Aeromonas hydrophila.*

culture is a contaminant, (2) the culture contained pigment and nonpigment producing forms, or (3) the original culture contained two organisms. In shipment the temperature could have been more favorable to the *Pseudomonas,* while in the laboratory a higher incubation temperature, if used, could have favored the *Achromobacter.* In the latter connection it is of interest to relate that a culture of *Pseudomonas* received from another source showed, in a smear, an occasional micrococcus. A subculture incubated at 35° C. had only micrococci; the *Pseudomonas* was apparently overgrown or killed at this temperature. On the other hand, only *Pseudomonas* grew in a subculture held at 5° C. Purification was obtained by several serial transfers held at 5° C.

In the case histories we have received and in those reported by others,[1, 4] the blood and autopsy cultures of recipients of pseudomonad contaminated blood were sterile but they were usually positive if a "coli-aerogenes" bacterium was the contaminant. In one history it is suggestive that the source of a "*B. coli*" surgical wound infection was from transfused blood. The patient was one of those that recovered from shock in the Bank E episode. We identified the culture from the blood that this patient received as *E. freundii.*

Although we have mentioned only gram-negative bacteria in connection with transfusion reactions, it should not be overlooked that gram-positive bacteria may also induce reactions. Five of the fourteen reactions collected from the literature by Borden and Hall[1] were caused by gram-positive bacteria, and we mentioned earlier the two reported by Braude and associates,[5] one caused by a diphtheroid and the other possibly by an enterococcus. Only one of the seven was fatal;[24] the causative organism was an anaerobic gram-positive coccus. However, since it appears from data available that contamination by gram-positive bacteria is more frequent than by gram-negative ones, it is reasonable to suggest that some of the milder febrile reactions following transfusion may be due to gram-positive bacteria. The relative pyrogenic activity of gram-positive and gram-negative bacteria has been studied by Probey and Pittman.[21]

Gram-negative Bacilli.—Thirty-two per cent of the 109 cultures studied were gram negative. Their distribution among the five products and their identification, with the exception of five, are given in Table II.

Pseudomonads and Unknowns: The significance of pseudomonads as contaminants of blood warrants presentation of the observed characteristics of our cultures. Any attempt to classify them as to species, no doubt, would only add

TABLE III. CHARACTERISTICS OF THE *Pseudomonas* STRAINS AND FOUR UNIDENTIFIED GRAM-NEGATIVE STRAINS

GENUS	STRAIN NO.	FLAGELLA*	PIGMENT†	GELATIN LIQ.	SERUM LIQ.	LITMUS MILK‡	USES		ACIDITY FROM§								
							CITRATE	ASPARAGIN	LACTOSE	MALTOSE	MANNOSE	RHAMNOSE	SUCROSE	ADONITOL	GLYCEROL	MANNITOL	SORBITOL
Pseudomonas	E‖	L	f	+	+	acpr	+	+	0	0	+	0	+	+	+	+	+
	113	L	f	+	+	a pr	+	+	0	0	+	+	−	+	+	+	+
	49	L	f	+	+	a pr	+	+	+	0	+	0	+	+	+	+	+
	84	L	f	+	+	a p	+	+	0	0	+	±	+	−	+	+	+
	25	L	f	−	−	a	+	+	0	0	+	0	−	0	−	−	0
	112	L	br	±	±	a p±	+	+	+	+	+	0	+	−	+	+	+
	4981¶	M	blue	+	+	acpr	+	+	0	0	+	0	−	0	+	+	0
	950**	L	y	−	−	a	+	+	0	0	+	0	−	0	−	0	0
Unknown	40	?	−	+	−	acpr	+	−	0	0	0	0	0	0	0	+	0
	57	?	−	+	+	Ac	+	−	±	0	+	0	0	0	0	0	0
	68	L	br	+	+	ac p	−	−	0	0	0	0	0	0	0	0	0
	101	?	cr	−	−	a	−	−	0	0	0	0	0	0	0	0	0

*L, lophotrichous; M, monotrichous; No. 101 was motile but flagella were not demonstrated; Nos. 40 and 57 were non-motile but both showed long fine threads, the majority of which were monopolar.

†f, soluble yellow-green fluorescent; br, brown; cr, cream; y, yellow.

‡A, a, c, p, r, indicate acid, alkaline, curdle, peptonization, reduction, respectively.

§All strains, except Nos. 68 and 101, produced acid from arabinose, glucose, galactose and xylose and no acid from raffinose, salicin, trehalose and dulcitol; +, ±, −, 0, indicate acid, slow acid production, no acid, no growth, respectively.

‖Five strains from Bank E episode.

¶Control, *P. aeruginosa* from Dr. E. Verder.

**Control, *P. ovalis*, from American Type Culture Collection.

to the existing confusion in the characterization of the species of this genus.[25, 26, 27] The morphologic and biochemical reactions are given in Table III; in the table are also given characteristics of two control pseudomonads and four unidentified strains. The sources of the cultures are recorded in subsequent Tables IV and V which give the growth temperature ranges.

Leifson's method[28] was used for flagella staining. Pigment production was observed on Gessard's glycerol agar slants for pyocyanin,[29] in asparagin solution containing magnesium sulfate and potassium hydrogen phosphate for green fluorescent pigment,[30, 31] and on tryptose agar slants containing 0.1 per cent glucose, in which the soluble-green pigment was particularly intense. The brown pigment of No. 112 was also observed on the latter medium; at first there was a slight darkening of the medium then, on storage at 5° C., the growth took on a reddish brown metallic appearance. The modified Ayers, Rupp, and Johnson ammoniacal medium used by Smith and Gordon[32] was employed, without agar, as the base for the carbohydrate media. A peptone base is not satisfactory for pseudomonads because of the alkaline reaction produced from the peptone.

The five pseudomonads cultures from the Bank E episode were identical, and their cultural reactions are recorded as a group in Table III. The other five pseudomonad strains differed from these and from each other. No. 25 was similar to control *P. ovalis* 950 except in pigmentation. The latter produced a nonsoluble yellow pigment. All differed from the control *P. aeruginosa* 4981 in that they were lophotrichous, did not produce pyocyanin, and grew at 2.5° C. but not at 41° C. (see Tables IV and V). Haynes[27] considers that ability to grow at 41° C. is one of several essential characteristics of *P. aeruginosa.* There is justification to conclude that none of our cultures were *P. aeruginosa.*

Of the unidentified cultures, Nos. 40 and 57 produced a thin iridescent film on the top of fluid media and No. 68 gave off an odor of ammonia. The fifth unidentified culture (No. 109), not listed in Table III, was isolated from influenza-virus vaccine. It was very pleomorphic, produced a coral pigment, was relatively inactive biochemically, and always grew slowly; the temperature range of growth was 10 to 34° C.

The Coli-Aerogenes Group: All of the Escherichia strains that we received were of the species *freundii,* none were of *coli.* In view of the fact that bacteria of *E. freundii* are capable of utilizing the salts of citric acid as the sole source of carbon, it may be that this characteristic, not possessed by *E. coli,* has some influence on the growth of *E. freundii* in blood, which is collected in the presence of a citrate. Another factor, however, may be involved, as two strains were also isolated from vaccines.

The six cultures listed as *P. aerogenoides* presented taxonomic problems. They were not all alike in morphology, in a few minor biochemical reactions, in growth temperature range, or in physiological reactivity, yet there was sufficient similarity to warrant placing them in one species. The two cultures from blood were very similar. One from plasma, No. 7,[21] was first thought to be *Aerobacter cloacae,* but later it was found to be capable of fermenting lactose after fourteen days. Another culture, No. 58, presented the greatest problem, and there is some evidence that it actually should be classified as *Aeromonas hydrophila.* In order to illustrate some of the identification problems encountered, some observa-

tions made on this culture will be presented. The main point in question has been the location of the flagella. In our preparations they were monopolar and predominately monotrichous, but in a preparation by Dr. S. Snieszko an occasional cell showed flagella arising laterally. He considered that the organism was peritrichous and that it should be classified as *Paracolobactrum.* The methods used for staining were Leifson's[28] and Novel's,[33] respectively. Bartholomew[34] has demonstrated both lateral and terminal origin of flagella on different cells in a single culture of *Pseudomonas.* The use of location of flagella as a criterion for classification is questionable. This is further complicated because of the general consideration that flagella are difficult to stain. The two cultures from blood, Nos. 114 and 119, were unquestionably peritrichous.

No. 58 also differed from these two blood cultures in that it grew at 41° C. and not at 5° C., while the reverse was true for Nos. 114 and 119 (Tables IV and V). Nos. 58 and 7 were tested for pyrogenicity. Both were active in inducing pyrexia in rabbits, but the former was also markedly toxic. A filtrate of a 3-day-old broth culture was lethal in a dose of 0.001 ml. per kilogram, while 0.00001 ml. per kilogram induced maximal fever response. (The pyrogen tests were carried out in collaboration with Mr. T. F. Probey.) It also induced fatal septicemia in mice. In pathogenicity, No. 58 resembled *hydrophila* which causes red leg in frogs and hemorrhagic septicemia in laboratory animals. The original source of this culture was traced to water. It caused the manufacturer considerable trouble in preparation of plasma before the source was found and measures for control were instituted. The proposed generic classification for *hydrophila* have been *Proteus,*[35] *Pseudomonas,*[36] and *Aeromonas.*[37, 38]

Gram-Positive Bacteria.—The Micrococcaceae: In Fig. 1, 44 strains of *Micrococcaceae* were recorded. The majority of these cultures were received in 1942. They were classified according to the species that they appeared to resemble most closely in the fifth edition of Bergey's Manual.[35] The 21 from plasma were distributed among 8 species as follows: 3, *Staphylococcus albus;* 9, *S. epidermidis;* 2, *Micrococcus epidermidis;* 1, *M. candidus;* 1, *M. flavus;* 3, *M. subflavescens;* 1, *S. aerogenes* (later classified as *M. prevotii* by Foubert and Douglas[39]) and 1, *Sarcina lutea.* The 21 from albumin and 2 from immune serum globulin were distributed among 3 species with the exception of 3 that did not fit into any species: 1, *S. albus;* 16, *S. epidermidis;* 1, *M. aurantiacus.* There were some variations between the organisms placed in a single species. When the cultures were grouped according to identical reactions, they fell into 18 groups: 10 groups had only 1 strain, 4 had 2, 1 had 8, and another 15. The only difference between the cultures in the last two groups was that the former hemolyzed blood rapidly while the latter did not or only slowly hemolyzed it; none fermented mannitol. These 23 cultures were classified as *S. epidermidis.* Seven of those that hemolyzed blood rapidly were from plasma, while 14 of those that hemolyzed blood poorly were from albumin.

What would have been the classification of these cultures according to the revision in the 6th edition of Bergey's Manual[36] is not known. It is evident, how-

ever, that there was considerable heterogeneity among the cultures, and that it was greater among the cultures from plasma. The latter would be expected if the source was from contaminated blood as was suggested earlier. With 14 of 21 cultures from albumin being identical, it appears that there was some natural selection. This might have been influenced by the high concentration of albumin (25 per cent) and sodium chloride (2 moles).

It is worthy of note that whereas *S. albus* has been reported to be the predominant micrococcus in contaminated blood, its relative incidence among our cultures was very low. It is also of interest to note that there was one anaerobic micrococcus among our strains. A death caused by an anaerobic coccus was mentioned above.[24] Foubert and Douglas[39] studied, besides our strain, 5 others that had been recovered from plasma.

The Streptococci: The 12 streptococci, isolated only from plasma, represented 5 species: 4, *S. liquefaciens;* 3, *S. faecalis;* and 5 in 3 unidentified species which did not fit into a type species as defined by Sherman.[40] Of the 4 *S. liquefaciens,* 2 were identical in biochemical activity, the others differed from these in that they did not peptonize milk, but they differed from each other in that one was not susceptible to phage D (No. 693, Evans[41]). The two identical strains were also susceptible to this phage. The nonsusceptible phage strain differed from the other 3 in not producing acid from glycerol. The 3 *S. faecalis* strains were alike in activity with the exception that one strain did not produce acid from raffinose. The other 5 strains were not tolerant to 6.5 per cent sodium chloride, pH 9.6, and 0.1 per cent methylene blue, although 2, which were identical, were capable of causing rapid reduction. The latter strains did not ferment lactose or mannitol. Two older identical strains also did not ferment lactose but they did ferment mannitol. The fifth strain did not ferment any carbohydrate; neither did it cause green discoloration of blood as did the other 11 strains.

The Bacillaceae and Diphtheroids: No particular effort was made to classify all of the 12 spore-forming strains beyond placing them in the genus *Bacillus.* Dr. Ruth Gordon identified 3 from plasma as *B. cereus.* One of these strains and another from plasma that we identified, rightly or wrongly, as *B. tumescens* showed a difference in the minimum temperature at which they grew. The *B. cereus,* No. 3A, was retarded in growth at 20° C. and did not grow at 10° C. whereas *B. tumescens,* No. 36, developed rapidly at 20° C. and more slowly at 10° C. (Table V).

The cultures designated as diphtheroids were identified on the basis of morphology, certain growth characteristics, and biochemical reactivity. Study for species identification was not done.

Growth Temperature Range of Bacteria.—The entrance of bacteria into a product is determined by the opportunities provided in the handling of the product, but the bacteria that develop after gaining entrance are selected on the basis of their ability to reproduce in the product. Factors that may influence reproduction are insusceptibility to any bactericidal activity of the product, ability to utilize the available nutrition and to reproduce at the temperatures at which the product is maintained or by error not maintained.

TABLE IV. GROWTH TEMPERATURE RANGE OF BACTERIA IMPLICATED IN HUMAN REACTIONS

BACTERIA	PROCESSOR	STRAIN NUMBER	GROWTH—°C.*							
			41	37	32	25	20	10	5-7	2.5
Pseudomonas	F	112†	–	–	–	+	+	2+	2±	5±
	B	113	–	–	+	+	+	2+	2±	5±
	A	84	–	–	+	+	+	2±	5+	5±
	E	117	–	–	+	+	+	2+	5±	5±
		115,116	–	±‡	+	+	+	2+	2±	5±
		120,121	–	3±	+	+	+	2+	3+	3±
P. aerogenoides	B	114	–	+	+	+	+	2+	5+	8±
	D	119	–	+	+	+	+	2+	5±	9±
E. freundii	E	118	+	+	+	+	+	4±	6±	–
		122,123	+	+	+	+	+	4±	–	–

*Presence of growth: –,none; ±, one tube; +, both tubes; no numeral and numerals indicate growth in 24 hours and the day it was observed, respectively.

†Slow growth was obtained at 30° C.

‡Growth from smaller inoculum: No. 115, in five days; No. 116, none in fourteen days.

We have observed the range of temperature at which 37 contaminants were capable of growing when other conditions were favorable. The cultures, grown on slants, were diluted in chilled distilled water to provide inocula of approximately 10^6 and 10^2 bacteria per milliliter, respectively, per tube of 15 ml. of fluid thioglycollate medium which was chilled at time of inoculation. A set of the two tubes was placed at temperatures ranging from 2.5 to 41° C. They were observed for visible growth at the end of twenty-four and forty-eight hours, and then at less frequent intervals for not less than twenty-one days. It was estimated that when visible growth could be detected that there had been at least a 10^2 and 10^6 fold increase in the number of bacteria in the respective tubes.

In Table IV are shown the temperatures at which the 13 bacteria, associated with human reactions, grew. They are listed in the order of ability to grow at lower temperatures and inability at the higher ones. The time when the first growth was noted is recorded. If growth developed in one tube at the lower temperatures it always developed in the other. This was not always true at the higher temperatures. It may be seen that all of the pseudomonads grew at least 100 fold at 2.5° C. in five days, growth was more rapid at 5° C. and 10° C., and most rapid at 20° C. and 25° C. They either did not grow or only poorly at 37° C. In fact some cultures were killed at 37° C. One culture did not grow at 32° C. It did grow slowly at 30° C. The cultures of *P. aerogenoides* also grew at 2.5° C. but more slowly. The cultures of *E. freundii* did not grow at 2.5° C., equivocally at 5°, but definitely at 10° C. They also grew at 41° C. whereas none of the others grew at this temperature.

In Table V are given the growth temperature range of 11 each of gram-negative and gram-positive bacteria isolated from the blood products and 2 control cultures. Each gram-negative strain from a blood product grew at 10° C. and 4 at 2.5° C. The control culture of *P. aeruginosa* did not grow below 10° C., and at this temperature growth from the larger inoculum was not observed until the tenth day; on the other hand, it grew rapidly at 41° C. in contrast to the failure of all of the pseudomonad contaminants. Growth at 41° C. is considered an essential characteristic of *P. aeruginosa*.

TABLE V. GROWTH TEMPERATURE RANGE OF BACTERIA ISOLATED FROM BLOOD PRODUCTS

BACTERIA	PRODUCT	STRAIN NUMBER	GROWTH—°C.							
			41	37	32	25	20	10	5-7	2.5
Pseudomonas (2 species)	Albumin	49	–	±*	+	+	+	2±	5+	5±
	Plasma	25	–	+	+	+	+	2±	5±	26±
A. faecalis		45	–	+	+	+	±	4±	8±	26±
E. freundii		23	+	+	+	+	+	4+	11+	15±
A. cloacae		3B,27	+	+	+	+	+	4+	–	–
P. aerogenoides		58	+	+	+	+	+	4+	–	–
Unidentified gram negative bacilli	Albumin	101	–	+	+	+	±	14+	–	–
		68	7+	+	+	+	+	7±	31±	–
	Plasma	40	+	+	+	+	+	4±	–	–
		57	±	+	+	+	+	4±	–	–
Micrococcus (5 species)	Plasma	13,14	+	+	+	+	+	7+	–	–
		8	+	+	+	+	5+	–	–	–
	Albumin	63	+	+	+	+	2±	–	–	–
	Plasma	17	+	+	+	+	5±	–	–	–
Sarcina lutea		110	+	+	+	+	2+	–	–	–
S. liquefaciens		16	+	+	+	+	+	7±	–	–
S. faecalis		29	+	+	+	+	±	4±	–	–
B. cereus		3A	+	+	+	+	±	–	–	–
B. tumescens		36	+	+	+	+	+	4±	–	–
A diphtheriod		18†	+	+	+	2±	11+	–	–	–
P. aeruginosa	Control	4981	+	+	+	+	±	10±	–	–
P. ovalis	Control	950	–	±	+	+	+	2±	2±	5±

See Table IV for explanations.
*Tube with smaller inoculum showed growth in seventeen days.
†At 30° C. growth was visible in both tubes in twenty-four hours.

The 11 gram-positive cultures selected for study required higher temperatures for growth than the majority of the gram-negative ones. All multiplied rapidly at 41° C. and all but one at 25° C. None grew at 5 to 7° C. but 5 grew at 10° C. within seven days. Two were micrococci, one *S. liquefaciens,* one *S. faecalis,* and one a sporeformer. The other 3 micrococci grew slowly even at 20° C. In the literature on blood, it is generally considered that gram-positive cocci and sporeformers do not multiply at refrigerator temperatures.

Near-optimum conditions probably prevailed for the growth of the bacteria in these experiments. In those bloods that have high bactericidal or inhibitory action, conditions for multiplication would not be as favorable. Bloods, however, differ in inhibitory action.[5] Our results show that if given a chance many of the common contaminants that may gain access to blood are capable of growing at storage temperatures used for blood and blood products; 10° C. is the maximum temperature permitted although 6° C. is recommended as the preferred maximum.[42]

DISCUSSION

The use of stored blood for transfusion has opened new fields for medical bacteriologists. Dairy bacteriologists have long been troubled with bacteria that grew at low temperatures, and now the frozen food bacteriologists are concerned with those that grow only in the icebox. Medical bacteriologists, accustomed to handling pathogenic bacteria, have not been fully aware that bacteria generally considered nonpathogenic are of first importance in the handling of

blood. The nonpathogens have a greater chance of gaining access to blood, and some have a lower growth temperature range. The latter multiply readily at temperatures used for blood storage and not only will not grow at 37° C. but are actually killed at this temperature. There has been concern about bacterial contamination from the donor's blood. Past experience indicates, however, that this source is relatively insignificant. Should a transient bacterium be in the blood of the healthy donor, it is believed that the chances are good, if the blood is kept properly refrigerated, that the bacterium will not multiply and will be of no more harm to the recipient than it was to the donor. One possible exception might be blood containing *S. albus* given to a child. A difference in pathogenicity of this organism for children and adults has been reported by Kotin.[43]

It is apparent that the bacteria that have grown in blood and caused severe and fatal transfusion reactions form a special group, largely composed of strains of certain species of *Pseudomonas* and of the coli-aerogenes group, all of which grow at blood-storage temperatures. The pseudomonads that we studied, in contrast to the enteric bacteria, grew more rapidly at lower temperatures and more poorly, if at all, at 37° C. The maximum for one culture was 30° C. It is questionable, however, that any could be called true psychrophiles as was indicated earlier.[44] The definition of psychrophilic varies in the textbooks. The fact that our cultures were not tolerant to 37° C. probably explains why MacFarlane and associates[17] had greater difficulty in obtaining sterile serum than plasma. They incubated the plasma at 37° C., whereas the serum was left at room temperature. The lack of tolerance for 37° C. also suggests one reason why negative cultures are obtained from the blood of recipients of pseudomonad contaminated blood.

Although our cultures of the coli-aerogenes group grew at the low temperatures, growth was not as rapid as with the pseudomonads, and in contrast they grew rapidly at 37° C. Further, they are usually recovered from the recipients. There are indications that certain of these cultures may induce, besides marked pyrexia, more profound circulatory disturbances.[1, 45] It appears from the case histories reported to us and in the literature that bacteria of *Paracolobactrum* may be the prime offenders. Slow fermentation of lactose separates it taxonomically from *A. aerogenes*. *E. freundii* is the only other organism of this group that we received. It differs from *E. coli* in that it is capable of utilizing citrate as the sole source of carbon. It may be of importance that all of our gram-negative cultures isolated from blood, both pseudomonads and enteric ones, were capable of utilizing this source of carbon, since it is present in bloods drawn for transfusion.

Although of less importance as blood contaminants, the gram-positive bacteria should not be neglected. The incidence of contamination is higher, their growth temperature range is higher, and they are less active in inducing pyrexia. It was found, however, that some of these contaminants from plasma were able to multiply at the upper temperature limit specified for blood storage.[42] All bacteria are apparently capable of inducing fever. The

intensity of the reaction, however, will vary with the kind and the number of bacteria injected.[21] It is not beyond reason to suggest that many of the mild reactions that follow transfusion are caused by the presence of gram-positive bacteria in blood. It is true that the lower grade reactions are not fatal but, as Whitby[46] stated, the reaction may be the last straw in an enfeebled patient, and often diminishes the benefit of the transfusion.

The indications that the bacterial contaminants of blood form a select group of nonpathogens suggests that emphasis should be placed on the temperature for the performance of the sterility test, in proper identification, and in choice of bacteria for studies relating to inhibitory action, preservatives, or other factors of blood. The temperature at which the sterility test is performed becomes of major importance. In our study the maximum temperature at which all cultures grew was 30° C., and the minimum was 20° C. One culture even grew slowly at 25° C. On the basis of the present information it appears that if only one temperature is to be used it should be between 25 and 30° C. The employment of a temperature in this range would necessitate, for summer months, the use of an incubator with a cooling device. One temperature, however, may favor the growth of one contaminant to the exclusion of others. Perhaps 37° C. incubation of pathogenic bacteria has favored the isolation and maintenance of pure cultures. Since some contaminants are killed at 37° C., a practice, of some, of starting the test at 37° C. and later placing it at a lower temperature should be abandoned. The true incidence of bacterial contamination of blood or blood products will not be known until a favorable temperature is used for the recovery of each contaminant. On the other hand, the relative incidence of bacterially caused transfusion reactions must await the application of procedures that take into account both the kind and quantity of bacteria present. These procedures should include steps to prevent both the multiplication of bacteria in the residuum of blood and the introduction of a new contaminant before the tests are made.

The most frequently mentioned bacterial contaminants in blood have been *E. coli*, *P. aeruginosa* (*pyocyaneus*) and *S. albus*. Among our 29 gram-negative rods and 44 micrococci from blood and blood products, *E. coli* and *P. aeruginosa* did not appear, and *S. albus* only 3 times. It is possible that contamination by these do occur, but it is believed that the number we studied is sufficient to indicate that their occurrence is not as frequent as has been considered generally. It appears that too often the contaminant has been designated by the more familiar name of a related organism. We found identification not easy and sometime impossible; perhaps some errors were made. Proper identification helps in tracing the source and thereby in instituting proper measures for control. It also aids in the selection of proper cultures for studies pertaining to blood contamination. The use of just any bacteria that happen to be in a collection may end in misleading conclusions. This study brings out both the importance of taxonomy, which has been sorely neglected, and the need for a good nomenclature key.

Molds, as contaminants of blood or blood products, have not been mentioned. This does not, however, belittle their possible significance in inducing transfusion reactions. Conditions that favor recovery of molds are seldom employed. They have been mentioned in the literature,[17, 18] and we have had verbal reports.

It is beyond the scope of this paper to recommend the techniques to be used to prevent contamination of blood. It would not be amiss, however, to emphasize that a select group of nonpathogenic bacteria are of prime importance and that rigid bacteriologic asepsis should be used. Surgical asepsis is not bacteriologic asepsis.[47]

SUMMARY

Eighteen severe or fatal reactions induced by bacterially contaminated blood or blood products are recorded. Thirteen cultures from the implicated or an associated product were studied. Eight were of *Pseudomonas,* none of which were *P. aeruginosa,* 2, *Paracolobactrum aerogenoides,* and 3, *Escherichia freundii.*

Eighty-five other cultures isolated from blood products, but not associated with reactions, were also studied. From both plasma and albumin, micrococci predominated; other cultures were gram-negative rods, sporeformers, and diphtheroids. Streptococci came only from plasma. The relative distribution of the cultures from plasma was similar to that reported for cultures isolated from blood. The micrococci from plasma represented a number of species, from albumin they comprised a greater percentage of the total but fewer species were represented.

The growth temperature range of all strains associated with reactions and of representative ones from plasma and albumin was determined. All strains associated with reactions and all other gram-negative rods grew at blood-storage temperatures. All pseudomonads grew at the lowest temperatures, some did not grow at 37° C., the others very poorly. The temperature range for the gram-positive bacteria, as a whole, was higher. A few, however, grew at 10° C., the upper limit for blood storage. The range in which all cultures grew readily was limited to above 25° C. and below 30° C.

The importance of nonpathogenic bacteria as the source of contamination of blood and blood products, the use of a proper temperature for the sterility test, and the correct identification of contaminants was discussed.

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Molds, as contaminants of blood or blood products, have not been mentioned. This does not, however, belittle their possible significance in inducing transfusion reactions. Conditions that favor recovery of molds are seldom employed. They have been mentioned in the literature,[17, 18] and we have had verbal reports.

It is beyond the scope of this paper to recommend the techniques to be used to prevent contamination of blood. It would not be amiss, however, to emphasize that a select group of nonpathogenic bacteria are of prime importance and that rigid bacteriologic asepsis should be used. Surgical asepsis is not bacteriologic asepsis.[47]

SUMMARY

Eighteen severe or fatal reactions induced by bacterially contaminated blood or blood products are recorded. Thirteen cultures from the implicated or an associated product were studied. Eight were of *Pseudomonas,* none of which were *P. aeruginosa,* 2, *Paracolobactrum aerogenoides,* and 3, *Escherichia freundii.*

Eighty-five other cultures isolated from blood products, but not associated with reactions, were also studied. From both plasma and albumin, micrococci predominated; other cultures were gram-negative rods, sporeformers, and diphtheroids. Streptococci came only from plasma. The relative distribution of the cultures from plasma was similar to that reported for cultures isolated from blood. The micrococci from plasma represented a number of species, from albumin they comprised a greater percentage of the total but fewer species were represented.

The growth temperature range of all strains associated with reactions and of representative ones from plasma and albumin was determined. All strains associated with reactions and all other gram-negative rods grew at blood-storage temperatures. All pseudomonads grew at the lowest temperatures, some did not grow at 37° C., the others very poorly. The temperature range for the gram-positive bacteria, as a whole, was higher. A few, however, grew at 10° C., the upper limit for blood storage. The range in which all cultures grew readily was limited to above 25° C. and below 30° C.

The importance of nonpathogenic bacteria as the source of contamination of blood and blood products, the use of a proper temperature for the sterility test, and the correct identification of contaminants was discussed.

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STUDIES IN THE DESTRUCTION OF STAPHYLOCOCCI BY HUMAN LEUKOCYTES: EFFECT OF CLUMPING OF INTRACELLULAR AND EXTRACELLULAR BACTERIA ON THE RESULTS OBTAINED WITH THE AGAR-PLATE METHOD

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THE activity of polymorphonuclear leukocytes in human staphylococcal infections is plainly seen by examining pus filled with leukocytes which have ingested staphylococci. The fate of the ingested staphylococci is unknown, however. There is evidence that phagocytosed bacteria are not necessarily dead. Studies of certain gram-negative bacilli have indicated that they may survive within leukocytes and even gain protection from bactericidal drugs or antibody.[1-4] Lyons,[5] and more recently Rogers and Tompsett,[6] suggested that phagocytosed pathogenic staphylococci also are capable of survival but that nonpathogenic staphylococci may be killed within human leukocytes. Fleming[7] reported that leukocytes were responsible for the killing of staphylococci by human blood.

Killing of bacteria can be determined quantitatively only by comparing viable counts made at different times. Two methods can be used. These are the dilution method and the plating method. The dilution method, which involves serial dilutions to the point at which no growth occurs, is cumbersome and subject to very large experimental errors.[8] The plating method, on the other hand, provides accurate counts under proper conditions. It is performed by adding the bacterial suspensions to a suitable solid medium and counting the colonies which develop after incubation. If agar is used, the bacteria may either be mixed with the melted agar or may be deposited on the solid surface.[9, 10]

As a means of determining whether human leukocytes can kill ingested staphylococci, we permitted staphylococci to incubate in mixtures of human plasma and leukocytes for varying periods. Then we determined the effect of such incubation on the number of bacteria which could be counted in plates of poured agar made with aliquots of the leukocyte suspension. We counted fewer colonies in those plates made with suspensions of leukocytes exposed to staphylococci for several hours than we counted in those plates made immediately after staphylococci were mixed with the leukocytes. These results suggested that human leukocytes killed staphylococci. This report describes later experiments, however, which disclose that the apparent reduction in the total number of staphylococci was an artefact resulting from aggregation

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This investigation was supported in part by a research grant from the National Cancer Institute, of the National Institute of Health, U. S. Public Health Service.
Received for publication, March 27, 1953.
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of the bacteria in the leukocyte after phagocytosis, from clumping of the phagocytes themselves, and from clumping of the bacteria in plasma outside the leukocytes. A clump of 2 or more cocci would appear in the agar plate as one colony.

Clumping of coagulase-positive staphylococci in plasma is a well-known phenomenon and serves as the basis for differentiating pathogenic from nonpathogenic strains of staphylococci.[11] Coagulase-producing staphylococci also clump in fibrinogen solution but not in serum. It appears, therefore, that fibrinogen is essential for clumping. Recently, it has been shown that pH affects the length of the intermediate polymer of fibrinogen. In strongly alkaline solutions with pH higher than 10, the length falls off so rapidly that the intermediate polymer does not form to any extent. The polymerization of fibrinogen is reversed above pH 10, and there is evidence that fibrin also will dissociate with increase of pH.[12, 13] In the following experiments, strongly alkaline solutions were used to break up the clumps of coagulase-positive staphylococci without materially affecting their survival. These alkaline solutions also appeared to be effective in disrupting leukocytes and releasing the enclosed staphylococci.

MATERIALS AND METHODS

Blood from normal persons was collected in sterilized rubber-stoppered bottles containing heparin in solution to give a final concentration of 0.5 mg. of heparin per milliliter of blood. The bottles had been partially evacuated so that venous blood could gently be drawn directly into each one by means of a sterile rubber tube with a needle at each end. This technique for obtaining blood, which avoided the use of a syringe, was found to produce minimal damage to leukocytes.

Leukocyte suspensions in plasma from normal persons were prepared by permiting the erythrocytes to sediment and removing the supernatant plasma with a warm sterile pipette. Supernatant plasma obtained in this way usually contained at least as many leukocytes per cubic millimeter as there were in the whole blood, and almost no erythrocytes. Sometimes it was necessary to centrifuge the supernate or the blood itself at 500 r.p.m. for five to ten minutes in siliconized glass tubes, to remove the erythrocytes. The leukocytes were sedimented only slightly at this speed of centrifugation.

Suspensions of leukocytes in serum were prepared by first separating the leukocytes from the plasma. This was done by centrifuging the plasma-leukocyte mixture at 1,000 r.p.m. for two minutes. The leukocytes collected in a layer at the bottom, and the plasma was removed with a pipette. These leukocytes were then resuspended in serum from the same donor. Suspensions of leukocytes in plasma, whose activity was to be compared with that of the serum suspensions, were also centrifuged at 1,000 r.p.m. for two minutes and the sedimented leukocytes resuspended in the supernatant plasma by gently tapping the tube. The number of leukocytes per cubic millimeter in the blood plasma and in the serum was determined before inoculating with staphylococci.

Cell-free plasma was prepared by centrifuging heparinized whole blood at high speed for twenty minutes. The supernatant plasma was removed and again centrifuged for twenty minutes. When necessary, serial dilutions of leukocyte suspensions were made with cell-free plasma. Each strain of pathogenic staphylococcus was isolated from human infections a few days before its experimental use. The organisms were identified as coagulase-positive both by their ability to clot heparinized human plasma in the tube and by clumping instantly when suspended in human plasma on a glass slide according to the methods described by Needham, Ferris, and Spink.[14] The coagulase-positive staphylococci were easily distinguished from the nonpathogenic staphylococci by the golden yellow color

and hemolysis produced on blood-agar plates. The strains of coagulase-negative staphylococci used in this study produced white colonies with little if any hemolysis on blood-agar; they were unable to clot plasma, and they did not clump when suspended in plasma.

In all experiments, staphylococci grown for eighteen hours in tryptose phosphate broth were resuspended in saline to give a density equal to that of the number 1 tube of the McFarland nephelometer. The suspension of staphylococci was then diluted in saline either to 10^{-6} or 10^{-2}, depending on whether heavy or light inoculums were desired. Because of the large errors involved in reaching a given dilution by pipetting, numbers of bacteria were selected for inoculation that could be counted in plates without serial dilution. Each milliliter of leukocyte-suspension in blood, plasma, or serum was inoculated with 0.2 ml. of staphylococcal suspension and incubated for six hours at 37° C. in siliconized tubes measuring 18 by 150 mm. The tubes were not agitated during incubation because every precaution was taken to avoid injuring the leukocytes. Immediately after removal from the incubator, the tubes were shaken to disperse the leukocytes and bacteria, and 3 aliquots of 0.5 ml. were removed. One aliquot was mixed with 2.0 ml. sterile distilled water of neutral pH, the second 0.5 ml. was mixed with 2.0 ml. of 0.1 per cent KOH, and the third was untreated. The 3 aliquots were incorporated into agar plates and incubated at 37° C. for twenty-four hours; the bacterial colonies were then counted. In all experiments, cell-free plasmas or serums were inoculated as controls and treated in the same way as were the suspensions of leukocytes. In a fourth aliquot, the number of leukocytes per cubic millimeter was determined before and after treatment with 0.1 per cent KOH and water. A Beckman pH meter was used for measuring pH.

EXPERIMENTAL

Experiment 1.—The purpose of this experiment was to determine the maximum activity of leukocytes before they were damaged by separation from whole blood. Samples of whole blood were obtained from donors and divided into equal portions. One portion was inoculated with a large number, and one with a small number, of coagulase-positive staphylococci (strain *BO*). Cell-free plasma from the same donor was inoculated as a control. The inoculated bloods and plasmas were incubated for six hours, and then pour plates were made.

Results.—Incubation of coagulase-positive staphylococci in whole blood for four to six hours invariably led to a decline in the number of bacterial colonies observed in agar-plates made with untreated aliquots. Staphylococci incubated in cell-free plasma, for the same period of time, however, appeared to increase in number when the plates were examined (Table I).

Treatment of the inoculated blood with KOH or water always resulted in much higher plate counts than were obtained with untreated aliquots. Treatment of aliquots with KOH after six hours of incubation sometimes gave higher plate counts than were obtained with inoculated blood before incubation. Treatment with KOH produced higher counts than water.

Treatment of incubated plasma with KOH also resulted in higher plate counts than were obtained with untreated plasma. Water did not increase the counts over those of the untreated plasma (sample 3).

The pH of whole blood in 0.1 per cent KOH was 10.4; the pH of plasma in 0.1 per cent KOH was 10.7.

Experiment 2.—The purpose of this experiment was to determine the effect of KOH and of water on bacterial counts when clumping due to the coagulase factor was eliminated by use of coagulase-negative staphylococci. A rise in plate count could be due only to dispersal of clumped intracellular cocci. Suspensions of leukocytes in plasmas

TABLE I. THE EFFECT OF KOH OR WATER ON PLATE COUNTS MADE TO DETERMINE THE BACTERICIDAL ACTION OF HEPARINIZED WHOLE BLOOD AGAINST COAGULASE-POSITIVE STAPHYLOCOCCI

SAMPLE OF BLOOD	LEUKOCYTES PER CUBIC MILLIMETER	HOURS OF INCUBATION OF STAPHYLOCOCCI IN BLOOD	PLATE COUNT OF BACTERIA PER MILLILITER OF BLOOD		
			NO KOH OR HOH	KOH	HOH
1	8,100	0	22,000		
		4	5,522		
		6	2,400	10,080	8,200
	Plasma O	0	672		
		4	80		
		6	42	380	260
2	5,400	0	21,000		
		4	25,000		
		6	Numerous	Innumerable	
	Plasma O	0	424		
		4	544		
		6	5,200	16,800	
3	5,600	0	20,400		
		4	6,600		12,000
		6	4,200	11,880	7,800
	Plasma O	0	496		
		4	116		
		6	58	422	180
		0	20,000		
		6	39,600	Innumerable	
		0	500		
		6	3,120	19,200	
	5,600	0	12,000		
		4	4,400	10,040	
		6	3,600	19,200	
	Plasma O	0	440		
		4	400	760	752
		6	272	2,800	780
		0	9,600	7,200	7,200
		6	40,000	Innumerable	32,000
		0	364	240	312
		6	9,840	20,000	7,550

The bacteria were counted in plates made with aliquots of inoculated blood which had been mixed with 0.1% KOH or water just before the plates were poured. The bacterial counts were compared with those obtained by pouring plates with unaltered aliquots of inoculated blood. Note the higher plate counts obtained when inoculated blood is treated with KOH or water or when inoculated plasma is treated with KOH.

from 7 donors, and the cell-free plasma controls, were divided into equal portions. One portion from each donor was inoculated with a strain of coagulase-positive staphylococcus, and the other portion with a strain of coagulase-negative staphylococcus. A total of 4 different strains of coagulase-positive and 6 different strains of coagulase-negative staphylococci were used. The inoculated suspensions and plasma controls from each donor were incubated simultaneously for six hours and then plates were made.

Results.—Incubation of coagulase-positive staphylococci with leukocytes in plasma for six hours led to a reduction in the number of bacterial colonies recovered in poured plates from untreated samples. Treatment with KOH always gave plate counts higher than those obtained with untreated aliquots, and sometimes the number of colonies in plates poured with KOH-treated

suspensions was greater after six hours than in plates made before incubation of the suspensions. In other words, the apparent bactericidal effect was nullified by addition of KOH. Treatment of the inoculated cell suspension with water also raised the bacterial count in many cases above that of the untreated specimen. In the case of cell-free plasma, all samples inoculated with coagulase-positive staphylococci yielded more colonies after six hours' incubation than at 0 hours, and treatment with KOH markedly increased the plate count over that of the untreated aliquot. Water had no significant effect on the plate counts obtained from inoculated plasma (Table II).

TABLE II. THE ACTION OF LEUKOCYTES SUSPENDED IN HEPARINIZED PLASMA ON COAGULASE-POSITIVE AND COAGULASE-NEGATIVE STAPHYLOCOCCI

PLASMA-LEUKOCYTE SUSPENSION		HOURS OF INCUBATION	PLATE COUNTS OF BACTERIA PER MILLILITER OF PLASMA							
			COAGULASE-POSITIVE				COAGULASE-NEGATIVE			
SPECIMEN NUMBER	W.B.C./C.MM.		STRAIN	UNTREATED	KOH	HOH	STRAIN	UNTREATED	KOH	HOH
1	15,000	0	BO	440			A	448		
		6		94	132	80		64	160	78
	0	0		712				1,568		
		6		2,400	5,040	2,300		6,000	5,040	4,800
2	6,150	0	BO	1,440			A	1,344		
		6		60	182	54		1,218	2,042	1,460
	0	0		660				1,280		
		6		992	2,520	1,154		6,600	6,000	6,600
3	11,000	0	BO	600			B	504		
		6		66	360	152		40	80	58
	0	0		540				728		
		6		660	6,240	876		256	204	198
4	6,900	0	GR	604			C	320		
		6		512	4,920	776		118	452	220
	0	0		778				318		
		6		2,080	20,160			368	356	
5	12,000	0	JO	2,160			D	420		
		6		832	1,920	1,020		85	544	280
	0	0		2,040				756		
		6		7,680	10,800			704	712	
	12,000	0	PA	1,560			E	608		
		6		396	1,440	556		348	398	384
	0	0		1,570				540		
		6		1,800	31,800			332	228	
6	9,100	0	BO	1,680			F	1,280		
		6		2,640	16,800	4,080		580	1,272	1,204
	0	0		1,360				1,240		
		6		9,940	19,200	10,560		2,400	2,640	2,600

Aliquots of the inoculated leukocyte-suspensions or of cell-free plasma were treated with KOH or water immediately before the plates were poured.

Incubation for six hours consistently reduced the number of coagulase-negative staphylococci recovered in plates made with untreated specimens. Hydroxide, and to a lesser degree water, increased the plate counts of coagulase-negative staphylococci from incubated cell suspensions but not from plasma. Such increases in plate counts were due to dispersal of clumped in-

tracellular cocci. Cell-free plasma sometimes reduced the number of coagulase-negative staphylococci which could be recovered after six hours' incubation.

The pH of leukocytes suspended in plasma after mixing with KOH was 10.8.

Experiment 3.—The purpose of this experiment was to determine the effect of KOH or water on bacterial counts when clumping due to the coagulase factor was eliminated by use of serum. Suspensions of leukocytes in serum and in plasma were made from blood obtained from 4 donors. Each suspension of cells was inoculated with a strain (BO) of coagulase-positive staphylococcus. Plates were made in the usual manner after six hours' incubation.

Results.—Plate counts made with incubated cell suspensions disclosed an increase in number of colonies in all cases except one (donor 4, plasma suspension), when compared to the counts obtained with suspensions which had not been incubated (Table III). These increases were undoubtedly related to the traumatic treatment of leukocytes during resuspension and were much less than those observed in the case of cell-free plasma. Treatment with KOH of all leukocyte suspensions, whether serum or plasma, always increased the yield of colonies over that of the untreated leukocyte suspensions. The increased yield obtained with water was less marked but consistent in the case of leukocytes suspended in serum or plasma.

The number of staphylococci appeared to increase in six hours in all cell-free plasmas and serums. This growth seemed to be much greater in serum, and the number of colonies was not increased by treating the inoculated serum

TABLE III. THE ACTION OF LEUKOCYTES SUSPENDED IN THEIR OWN SERUM AND HEPARINIZED PLASMA ON A STRAIN OF COAGULASE-POSITIVE STAPHYLOCOCCUS

ORIGIN OF LEUKOCYTES: DONOR NUMBER	HOURS OF INCUBATION	PLATE COUNTS OF BACTERIA PER MILLILITER							
		PLASMA				SERUM			
		W.B.C. PER C.MM.	UNTREATED	KOH	HOH	W.B.C. PER C.MM.	UNTREATED	KOH	HOH
1	0	4,300	952			3,650	780		
	6		1,106	11,000	1,518		3,000	10,800	4,040
	0	0	1,032			0	1,020		
	6		7,560	21,600	7,440		24,000	26,400	21,600
2	0	8,000	1,200	1,440		11,500	1,560		
	6		1,920	17,400	2,800		1,800	2,940	2,400
	0	0	1,440			0	1,800		
	6		24,400	I			27,200	28,000	
3	0	9,100	1,680			6,100	1,440		
	6		2,640	16,800	4,080		5,280	10,360	8,400
	0	0	1,360			0	1,538		
	6		9,840	19,200	10,560		20,400	20,000	20,400
4	0	7,500	1,304			7,200	1,560		
	6		760	1,440	920		2,640	4,800	3,360
	0	0	1,360			0	1,400		
	6		6,400	14,000	7,000		14,400	15,360	13,000

Aliquots of the inoculated leukocyte-suspensions and of cell-free plasma or serum were treated with KOH or water immediately before the plates were poured.

I = innumerable.

with KOH or water. Treatment of inoculated plasmas with KOH increased the number of staphylococci approximately to the number observed in plates made with the corresponding sample of serum. Water had no significant effect.

The pH of the serum-leukocyte suspension in KOH was 10.6, and the plasma-leukocyte suspension in KOH was 10.8.

The results are summarized in Table III.

Experiment 4.—Leukocytes were diluted serially in their own plasma to give a series of suspensions containing the number of cells indicated in Table IV. Each suspension was inoculated with coagulase-positive staphylococci (strain BO) and incubated for six hours. Pour plates were made at the end of the six hours.

The purpose of this experiment was to observe the relative effect of KOH and water on plate counts made with suspensions containing decreasing numbers of leukocytes. The higher plate counts obtained by treatment with KOH and water are expressed in Table X as per cent rise over the counts obtained with untreated aliquots.

Results.—The effectiveness of water and KOH in producing higher plate counts of staphylococci incubated in leukocyte suspensions for six hours was again observed as in the previous experiments. This effect of water gradually diminished and was finally lost as the leukocyte count of the suspension fell to zero. The effect of KOH, on the other hand, was maintained in the absence of leukocytes (Table IV).

DISCUSSION

These results indicate that three factors lead to errors in the usual bactericidal test when coagulase-positive staphylococci are incubated in fresh human blood and counted in pour plates. One factor is undoubtedly clumping of the staphylococci in the presence of fibrinogen, and such clumping can be prevented by raising the pH to 10.2 or higher. The other factors seem to be related to phagocytosis. The second factor is aggregation of bacteria within leukocytes, and the third is clumping of the phagocytes containing ingested bacteria. This clumping of staphylococci resulting from the presence of leukocytes can also be prevented by raising the pH of the suspending menstruum or by diluting it with distilled water. The effect of clumping, regardless of the cause, is to decrease the bacterial count in pour plates below its true value.

Several lines of evidence support the premise that the coagulase factor leads to clumping during the bactericidal tests described. Increase of pH of plasma or blood to 10 or higher will increase the number of colonies of coagulase-positive staphylococci appearing in agar plates, but the number of colonies recovered from serum or broth is unaffected by the same change in pH. Similarly, the plate counts obtained with plasma inoculated with coagulase-negative staphylococci are the same whether the pH is neutral or alkaline. It is known that the length of the intermediate polymer of fibrinogen is sharply reduced at pH levels above 10 and that clumping of coagulase-producing staphylococci depends on the presence of fibrinogen. By raising the pH of plasma to 10.5 we have been able to disrupt grossly visible clumping of coagulase-positive staphylococci which appeared with a heavy inoculum. Microscopically, innumerable dispersed cocci were seen in the alkaline plasma.

It is of particular interest that results of the usual plate method indicated that a given strain of coagulase-positive staphylococcus reached greater numbers after six hours' growth in serum than in plasma derived from the same blood. Increase in pH of the plasma to 10.4, however, led to plate counts equal to those obtained with the serum. In other words, it may be possible that clumping related to coagulase production can be completely disrupted at the proper pH.

The apparent diminution in number of staphylococci observed after they are incubated with leukocytes is probably due, in part, at least, to phagocytosis and intracellular clumping. When human leukocytes which had ingested staphylococci were microscopically examined in wet preparations to which KOH was added, they were seen to rupture at a point on their surface through which the bacteria escaped into the surrounding medium. It is not surprising, therefore, that when coagulase-negative staphylococci were incubated in plasma-leukocyte suspensions, higher plate counts were obtained if suspensions had been treated with KOH than if they were untreated. Likewise, the counts were increased by adding KOH to coagulase-positive staphylococci incubated

TABLE IV. RELATIONSHIP OF NUMBER OF LEUKOCYTES IN PLASMA TO THE NUMBER OF COAGULASE-POSITIVE STAPHYLOCOCCI CULTURED AFTER SIX HOURS' INCUBATION

PLASMA LEUKOCYTE-SUSPENSION		HOURS OF INCUBATION	PLATE COUNTS OF BACTERIA PER MILLILITER				
DONOR NO.	W.B.C. PER C.MM.		UNTREATED	KOH	% RISE	HOH	% RISE
1	20,100	0	1,680				
		6	232	616	165	432	86
	10,150	0	1,920				
		6	602	5,400	979	1,064	77
	5,950	0	1,780				
		6	1,560	14,440	825	3,000	92
	2,250	0	2,060				
		6	8,880	I		12,840	44
	1,400	0	2,280				
		6	17,520	I		19,000	8
	0	0	1,800				
		6	36,000	*		*	
2	16,250	0	1,440				
		6	332	1,800	442	538	62
	8,100	0	1,560				
		6	740	4,800	549	1,080	46
	4,400	0	1,360				
		6	6,400	17,000	107	8,160	27.5
	1,850	0	1,440				
		6	5,760	14,400	166	6,000	4.2
	0	0	1,700				
		6	24,000	I		23,000	0

Serial dilutions of leukocyte-suspension were made in their own cell-free plasma. Aliquots of the inoculated leukocyte-suspensions were treated with KOH or water immediately before the plates were poured. The higher plate counts obtained by such treatment were expressed as % rise over the counts obtained with untreated aliquots.

I = innumerable.

*Not determined.

in serum-leukocyte mixtures. Coagulase was not a factor in either case, and disruption of extracellular clumps of cocci could not have occurred, since the counts of the control sera or plasma were not changed by similar rise in pH. The best explanation for these increases in plate counts is rupture of the leukocytes and dispersal of their phagocytosed cocci. Rupture of leukocytes can also be achieved by exposing them to water without change in pH. The rise in plate count after exposure to water is consistently less than after exposure to KOH. Exposure of leukocytes to water was useful, however, for demonstrating intracellular clumping of coagulase-positive staphylococci in plasma suspension, because water did not break up the extracellular clumps of cocci which developed from coagulase activity (Table IV).

The agar-plate method must be regarded as unsatisfactory for determining whether leukocytes will kill staphylococci, because the apparent number of viable bacteria may be reduced through other means than killing. The errors can be partly corrected by treating the suspension of leukocytes and bacteria with KOH before making the agar plates. But it is unlikely that bacterial clumping can be overcome sufficiently to render the agar-plate method valid for measuring the bactericidal power of leukocytes.

There are two other sources of error in estimating numbers of staphylococci by the plating method. These are the dilution error and the distribution (sampling) error. The dilution error results from pipetting, and increases with the number of transfers needed to reach a given dilution. We avoided the dilution error by working with numbers of bacteria which could be counted in plates without serial dilution. The distribution error is not large. According to Jennison, the coefficient of variation between replicate plates under good experimental conditions is only ± 4 to 5 per cent.[9]

Rogers and Tompsett[6] found that in the presence of human leukocytes in plasma there was a rapid fall in the numbers of culturable staphylococci in pour plates of both coagulase-positive and coagulase-negative strains. They attempted to disrupt intracellular aggregates by grinding sand suspensions of leukocytes which had been incubated with staphylococci up to three hours. They found, however, no significant difference in the number of culturable staphylococci obtained in pour plates from the ground and unground samples. Examination of their data, however, discloses that after longer periods of incubation, when the coagulase-positive staphylococci themselves had disrupted the leukocytes, the plate counts rose quickly above those obtained with plasma controls containing no leukocytes. Disintegration of leukocytes containing coagulase-negative staphylococci occurred more slowly so that subsequent increases in plate counts took place later than those observed with coagulase-positive staphylococci. The authors attributed the differences in time of resurgence of plate counts to differences in the ability of coagulase-positive and coagulase-negative staphylococci to survive within leukocytes. They did not mention the possibility that the rise in plate counts resulted from a dispersal of intracellular aggregates following destruction of the phagocytes.

SUMMARY AND CONCLUSIONS

The apparent reduction, observed by the plate method, in numbers of coagulase-positive staphylococci after incubation in human blood may result from clumping of living bacteria in leukocytes and in plasma or from clumping of phagocytes containing living bacteria. A clump of 2 or more cocci would appear as one colony in the agar pour plate and give the erroneous impression that the bacteria had been killed.

The clumping of staphylococci may be disrupted by raising the pH of the inoculated blood above 10 at the time the plates are poured. The high pH disperses phagocytosed clumps of cocci by disrupting leukocytes and also breaks up extracellular clumps of cocci. The extracellular clumps of coagulase-positive staphylococci in plasma appear to depend on the presence of fibrinogen. Removal of fibrinogen from plasma prevents clumping of coagulase-positive staphylococci. Increasing pH above 10, which is known to reduce the length of the intermediate polymer of fibrinogen, disrupts the clumps of coagulase-positive staphylococci in plasma.

It is unlikely that bacterial clumping can be overcome sufficiently to render the agar-plate method valid for measuring the bactericidal power of leukocytes.

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STUDY OF MOVEMENTS OF CUTANEOUS INTERSTITIAL FLUIDS IN HUMAN BEINGS BY MEANS OF A FLUORESCENT TRACER SUBSTANCE*

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NO PRACTICAL method has been available for the determination of the rate at which interstitial fluid is removed from the tissue spaces of human skin. It has been assumed that the rate of removal of such fluid depends on the flow of blood and lymph. Cutaneous flow of blood has been estimated by finger plethysmography or determination of cutaneous temperature. Flow of lymph in the skin has been estimated by cannulization of subcutaneous lymphatic channels[4, 5] or by the rapidity with which dyes fill the lymphatic capillaries.[7, 12] McMaster[13] has used micropipettes to study the flow of interstitial fluid in the skin of animals, but the method is not readily applicable to human beings.

In this study an attempt has been made to develop a suitable and practical means to study the flow of interstitial fluid in the skin of human beings, with the hope that determination of the rate of absorption of interstitial fluid would shed new light on the pathophysiology of various types of edema.

It appears rational to consider that the rate of disappearance of a tracer substance injected into tissue would be an indication of the rate at which the tissue fluid was being removed. Various tracer substances have been used. McMaster[12] injected intradermally the dye patent blue V and noted the rapidity with which it spread along the lymphatic channels. He considered this an indication of cutaneous lymphatic flow. Kety[8, 9] and others[3, 6] have injected radiosodium into the gastrocnemius muscle of human beings and have measured its disappearance with a Geiger counter. The rate of disappearance of sodium[24] was assumed to reflect the regional circulation in the muscle. Wisham and Yalow have shown that radiosodium disappears much more rapidly after intramuscular or intradermal injection than after subcutaneous injection.

The present study is concerned with the photofluorometric determination of the rate of disappearance of a fluorescent tracer substance after its intradermal injection. Zehnder has reported that fluorescein disappeared more rapidly from the skin of persons who were given hypertonic solutions of glucose. However, he did not use a photoelectric apparatus to make quantitative measure-

Received for publication, Mar. 16, 1953.

*Abridgment of thesis submitted by Dr. Gifford to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

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ments. No other reports have been found in the literature concerning the use of a fluorescent tracer substance as a means of studying the flow of cutaneous interstitial fluid in human beings.

MATERIALS AND METHODS

The synthetic sodium salt of riboflavin (Flavaxin*) was used as the fluorescent tracer substance in this study. Under ultraviolet light, solutions of riboflavin exhibit a brilliant yellow-green fluorescence. The commercially available powder for parenteral administration was dissolved in 0.9 per cent solution of sodium chloride so that each cubic centimeter contained 2.5 mg. of the sodium salt of riboflavin.

All injections were intradermal, and 0.02 ml. of the solution of riboflavin was given in every instance. To deliver accurately such small quantities, a syringe patterned after that described by Burch was constructed (Fig. 1). To minimize the trauma of injection, well-sharpened, 27-gauge, 0.5-inch (1.27-cm.) hypodermic needles were used.

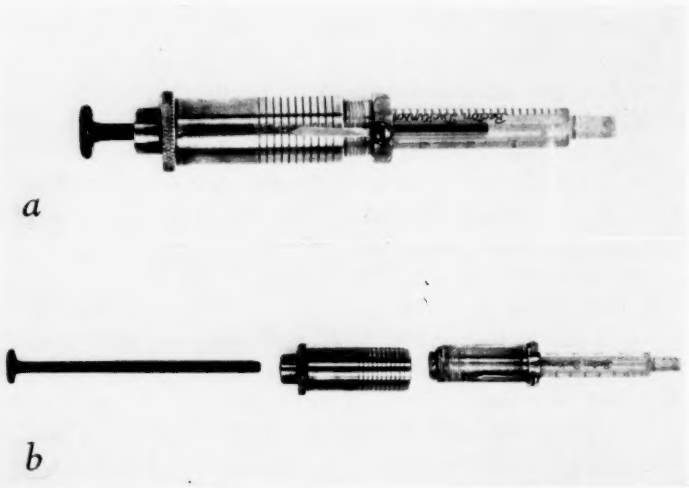

Fig. 1.—Special syringe used to deliver minute but easily reproducible volumes of solutions of riboflavin. *a.* Assembled syringe. *b.* Plunger has been withdrawn and outer metal cylinder removed.

The photofluorometer used to measure the intensity of fluorescence of riboflavin after its injection into the skin was a modification of the instrument described by Minard and Eicher.[15, 16] A similar apparatus has been described by Schiller.[18]

To determine the intensity of fluorescence of the injected solution of riboflavin, the vertex of a portable V-shaped holder (Fig. 2) was applied over the injected portions of skin. The aperture at the vertex of the V was approximately 2.5 cm. in width. At the upper end of one arm of the V-shaped holder was the ultraviolet source, a G.E. F5000 mercury-vapor lamp. The ultraviolet light emitted from this lamp was directed down the limb of the V to the skin at the vertex, where it excited fluorescence. The reflected fluorescent light was directed against an RCA 931-A photomultiplier tube located at the upper end of the other limb of the V-shaped holder. A No. 5970 Corning-glass filter was used to filter the incident rays, while a No. 3480 Corning-glass filter was used to filter the reflected light.

The V-shaped holder was easily maneuvered and could be applied in rapid succession to various parts of the skin where the intensity of fluorescence was to be determined. Although

*Winthrop-Stearns, Inc., New York, N. Y.

all readings were taken in subdued light, it was still essential to insure a lightproof seal between the instrument and the skin.

The entire apparatus was made portable by placing it on a surgical dressing cart (Fig. 3). The metal containers on the lower shelf housed the circuits for the photomultiplier tube and the ultraviolet lamp. The dynode supply came from nine Burgess type N-60 batteries, each furnishing 90 volts. A 45-volt battery furnished the plate supply, while a 22.5-volt battery furnished the bucking voltage necessary to reduce deflection of the meter to zero. Readings were made directly from a 0-microampere to 50-microampere Triplett meter that could be shunted for sensitivities of 50, 100, 250, 500, and 1,000 microamperes full-scale deflection. A combination switch for meter sensitivity and off-and-on bucking voltage was used. The ultraviolet lamp was supplied by a 24-volt storage battery that was charged from house current whenever the instrument was used.*

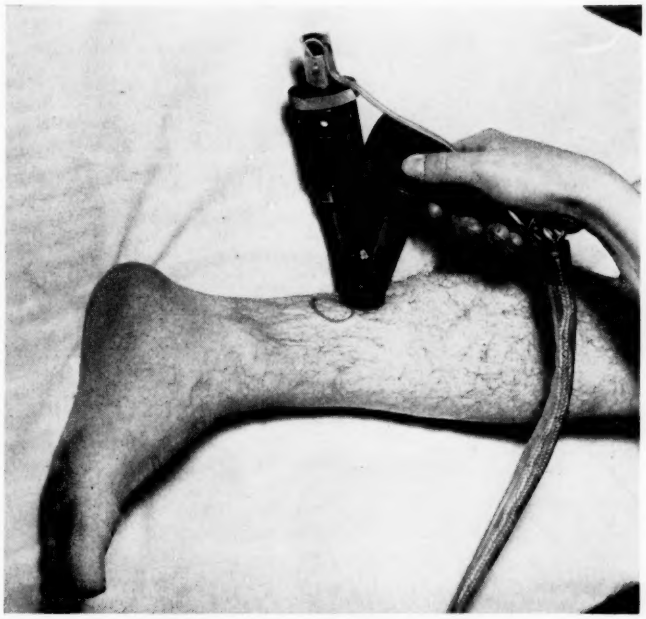

Fig. 2.—Portable V-shaped holder used to make photofluorometric readings on the skin. The site of injection of riboflavin is shown.

PROCEDURES

Normal persons were first studied in the supine position in a room of constant environmental temperature (25.5° C.). The persons were denied drugs, tobacco, and food for at least six hours before and during each procedure.

Whenever feasible, cutaneous temperatures of the toes were recorded throughout the procedure by means of copper-constantan thermocouples.

The studies were limited to the skin of the legs. Injections were made in the skin on the medial surface of the leg about 3 cm. proximal to the internal malleolus (Fig. 2). Two injections were usually made in each leg and the readings from the two regions were averaged to give a single value for each leg.

*The authors gratefully acknowledge the technical assistance of Martin Jacobs and Merlin Neher in the assembly and maintenance of the photofluorometer and micro-syringe used in these studies.

RESULTS

After the intradermal injection of riboflavin, the intensity of fluorescence at the site of injection was measured by the photofluorometer every five or ten minutes. After the injection, the intensity of fluorescence in the skin increased to a maximal value, usually within the first twenty minutes. Then the intensity of fluorescence gradually decreased during a period of four to five hours. Often traces of fluorescence, too small to be detected by the photofluorometer, could be seen six hours after injection; thus it was usually difficult if not impossible to determine accurately the time when the riboflavin had completely disappeared. To obviate this difficulty, and at the same time to shorten the time of

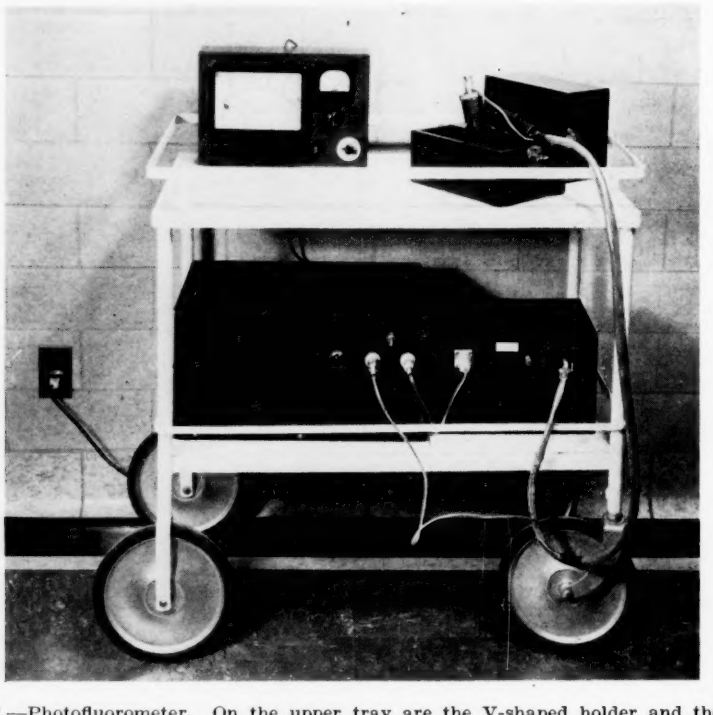

Fig. 3.—Photofluorometer. On the upper tray are the V-shaped holder and the Triplett meter. Boxes on the lower tray house the circuits for the source of ultraviolet light and for the photomultiplier tube.

each observation, the time was measured from injection of the riboflavin until the intensity of fluorescence had diminished to half the maximal value. This time expressed in minutes was designated as the "50 per cent time." Long 50 per-cent times indicate slow absorption of the riboflavin, while short 50 per-cent times indicate rapid absorption.

Normal Persons Under Basal Conditions.—The 50 per-cent time was determined in the skin of forty legs of 20 normal volunteers who had no histories or physical findings of circulatory disorders (Table I). Fourteen of the subjects were women and 6 were men. No significant differences were noted between the left and right legs of any single person at any one time nor did any differ-

TABLE I. DISAPPEARANCE OF RIBOFLAVIN FROM SKIN OF LEGS OF 20 NORMAL PERSONS UNDER BASAL CONDITIONS

50 PER-CENT TIME, MINUTES			S.D.* OF MEAN	S.E.† OF MEAN
MAXIMAL	MINIMAL	MEAN		
244	74	148	32	±5.1

*S.D. = standard deviation (also in Table II).
†S.E. = standard error (also in all other tables).

ence exist between findings in the sexes. Repeated determinations on different days were done in 3 volunteers; 2 exhibited remarkably constant values from day to day, while values in the third varied greatly (Table II).

Normal Persons Under Abnormal Conditions.—In order to learn whether or not the rate of disappearance of riboflavin would parallel changes in the flow of tissue fluid, the effects of temperature, dependency, venous obstruction, and certain drugs on the disappearance of riboflavin were studied. In all of the following studies paired data were obtained by a comparison of the results noted in each leg under experimental conditions to those results previously obtained in the same leg under basal control conditions. The mean of the differences and its standard error were calculated, and from this the value of P was computed to indicate the significance of any difference obtained.

TABLE II. REPEATED DETERMINATIONS OF DISAPPEARANCE OF RIBOFLAVIN FROM THE SKIN OF LEGS OF 3 NORMAL PERSONS UNDER BASAL CONDITIONS

PERSON	DETERMINATIONS*	50 PER-CENT TIME, MINUTES			S.D. OF MEAN	S.E. OF MEAN
		MAXIMAL	MINIMAL	MEAN		
1	5	190	68	106	48.4	±22
2	5	154	115	127	9.4	± 4.3
3	4	149	127	138	5.8	± 2.9

*Done on different days.

Effect of Environmental Temperature on Disappearance of Riboflavin.—Warm Environment: Six persons were studied in a thermostatically controlled hot room, where a constant temperature of 30° C. was maintained. Except for the increased environmental temperature, all other basal conditions remained unchanged. The cutaneous temperatures of the toes of these persons were significantly increased, and in eleven of the twelve legs the 50 per-cent time decreased from the control values obtained in a room at 25.5° C. The mean decrease was twenty-six minutes, which was statistically significant (Table III).

Cool Environment: Six persons were studied in a thermostatically controlled cold room, where a constant temperature of 20° C. was maintained. The

TABLE III. EFFECT OF ENVIRONMENTAL TEMPERATURE ON DISAPPEARANCE OF RIBOFLAVIN FROM SKIN OF LEGS OF NORMAL PERSONS

ENVIRONMENTAL TEMPERATURE, °C.	LEGS	CUTANEOUS TEMPERATURE (°C.), MEAN AND S.E.	P*	50 PER-CENT TIME (MINUTES), MEAN AND S.E.	P*
25.5	12	28.6 ± .6		127 ± 7.2	
30.0		34.2 ± .65	<.01	101 ± 8.7	<.01
25.5	12	27.8 ± .9		133 ± 8.6	
20.0		20.0 ± .3	<.01	274 ± 18	<.01

*Significance of difference.

cutaneous temperatures of the toes of each person were decreased from the temperatures obtained in the room at 25.5° C., and all but one of the legs showed an increase in the 50 per-cent time over the values obtained in the room at 25.5° C. The maximal increase was 208 minutes, and the mean increase 141 minutes (Table III).

Effect of Dependent Position on Disappearance of Riboflavin.—Studies With Persons in Sitting Position: Seven persons were studied while they were in a sitting position. Except for the dependent position of the legs, all other basal conditions remained unchanged. As a crude approximation of venous pressure,[17] the height of the venous column was determined by a vertical measurement from the level of the third costal interspace at the sternum to the level of the sites of injection. Under these conditions the 50 per-cent time was prolonged in all legs, the maximal increase being 132 minutes. The average increase was seventy-five minutes; the difference was statistically significant (Table IV).

TABLE IV. EFFECT OF DEPENDENT POSITION ON DISAPPEARANCE OF RIBOFLAVIN FROM SKIN OF LEGS OF NORMAL PERSONS

POSITION OF PERSON	LEGS	MEAN HEIGHT OF VENOUS COLUMN (CM.)	50 PER-CENT TIME (MINUTES), MEAN AND S.E.	P*
Supine	14	0	133 ± 6.2	
Sitting		66	208 ± 10.6	<.01
Supine	10	0	134 ± 7.3	
Walking		113	178 ± 15.6	<.01

*Significance of difference.

Effect of Dependency of Legs Plus Muscular Activity on Disappearance of Riboflavin: Five persons walked throughout this part of the study. The venous columns from the cardiac level to the sites of injection were measured, but it must be remembered that the venous pressure in a walking person is less than that in one who is stationary, even though the venous columns are the same height.[17] The 50 per-cent time was prolonged in all but one leg. The average increase was forty-three minutes, which was statistically significant (Table IV).

Effect of Venous Occlusion on Disappearance of Riboflavin.—The cuff of a sphygmomanometer was applied to a thigh in 5 supine persons; the cuff was inflated to a pressure between 40 and 50 mm. of mercury before any injections were made. The opposite leg served as a control; all other basal conditions were unchanged. The 50 per-cent time was prolonged in the leg occluded by the cuff in all 5 persons. In 1 person it was increased by 117 minutes over the opposite leg. The average increase was sixty-three minutes, which was a statistically significant increase (Table V).

TABLE V. EFFECT OF VENOUS OCCLUSION ON DISAPPEARANCE OF RIBOFLAVIN FROM SKIN OF LEGS OF NORMAL PERSONS

LEGS		50 PER-CENT TIME	
CONDITION	NUMBER	(MINUTES), MEAN AND S.E.	P*
Control	5	144 ± 18.9	
Inflated cuff on thigh	5	207 ± 15.6	0.01 to 0.02

*Significance of difference.

Effect of Drugs on Disappearance of Riboflavin.—Histamine: Five persons were studied under the basal conditions described previously except that just prior to the injection of riboflavin two intradermal wheals were raised in the leg at the usual sites of injection of riboflavin by injection of 0.1 ml. of histamine diphosphate (0.275 mg. per ml.). Control wheals were raised in the opposite leg by injection of 0.1 ml. of 0.9 per cent solution of sodium chloride after it had been established that such wheals produced no change in the rate of disappearance of riboflavin. Into each of the wheals the usual injections of riboflavin were made immediately. The 50 per-cent time was shortened greatly in all sites previously injected with histamine as compared to the control sites injected with solution of sodium chloride in the opposite leg. This decrease in 50 per-cent time was as great as 101 minutes in 1 of the persons. The mean decrease was eighty-two minutes, which was statistically significant (Table VI).

Epinephrine: Five persons were studied under basal conditions except that intradermal wheals of 0.1 ml. of 1:1,000 solution of epinephrine were raised in one leg, while wheals of 0.9 per cent solution of sodium chloride were raised in the skin of the opposite leg as controls. Into these wheals the usual injections of riboflavin were made immediately. The 50 per-cent time was greatly prolonged in all sites into which epinephrine had been injected previously as compared to the 50 per-cent time of the control sites. This increase ranged from 127 to 239 minutes, and averaged 180 minutes (Table VI). The statistical significance is beyond doubt. The largest 50 per-cent time encountered in the entire investigation was recorded from one of the legs into which epinephrine had been injected previously.

TABLE VI. EFFECT OF LOCALLY INJECTED SOLUTIONS ON DISAPPEARANCE OF RIBOFLAVIN FROM SKIN OF LEGS OF NORMAL PERSONS

SOLUTION INJECTED	LEGS	50 PER-CENT TIME (MINUTES), MEAN AND S.E.	P*
Saline	5	131 ± 9.1	
Histamine	5	49 ± 13.2	<.01
Saline	5	136 ± 10.7	
Epinephrine	5	316 ± 20.5	<.01

*Significance of difference.

Patients Who Had Peripheral Vascular Diseases.—In a third phase of this investigation, the rate of disappearance of riboflavin from the skin of the legs of 20 patients who had a variety of circulatory disorders was studied. Values obtained in the legs of the normal persons under basal conditions were used as controls.

TABLE VII. EFFECT OF CHRONIC OCCLUSIVE ARTERIAL DISEASE ON DISAPPEARANCE OF RIBOFLAVIN

	LEGS	50 PER-CENT TIME (MINUTES), MEAN AND S.E.	P*
Normal persons	32	140 ± 6.0	
Patients who had occlusive arterial disease	11	146 ± 8.8	.5 to .6

*Significance of difference.

Chronic Occlusive Arterial Disease Without Edema.—Eleven legs of 6 patients who had chronic occlusive arterial disease were studied. Although all the legs studied were extensively ischemic, the 50 per-cent times were within the range of values determined in the controls (Table VII).

Disorders With Peripheral Edema.—In Table VIII are shown the various disorders considered, together with the number of legs studied and the results. It is remarkable that in all of these circulatory disorders, which usually are associated with edema, a tendency was noted for more rapid disappearance of the riboflavin than occurred in normal persons used as controls. However, only in patients who had simple orthostatic edema was a statistically significant decrease noted in the mean when compared to the mean of the normal persons.

TABLE VIII. EFFECT OF THROMBOPHLEBITIS, ORTHOSTATIC EDEMA AND LYMPHEDEMA ON DISAPPEARANCE OF RIBOFLAVIN

		PERSONS	LEGS	50 PER-CENT TIME (MINUTES), MEAN AND S. E.	P*
Normal		16	32	140 ± 6.0	
Disease	Acute iliofemoral thrombophlebitis	2	3	110 ± 12	.02 to .05
	Simple orthostatic edema	5	10	85 ± 6.1	<.01
	Lymphedema	7	8	112 ± 14.7	.05 to 0.1

*Significance of difference.

It was further noted that when repeated determinations were made on edematous legs, the 50 per-cent time often remained rapid even though the edema disappeared under treatment. In 3 of the 7 patients who had unilateral edema, the riboflavin disappeared much more rapidly from the edematous leg; the riboflavin was retained longer in the edematous leg in only 1 of the 7.

For this reason, the 50 per-cent times obtained in all twenty-one of the extremities that were edematous or potentially edematous were analyzed according to the degree of edema as estimated clinically (from grade 0 to grade 4 in which 0 represents absence of edema, 1 the least amount, and 4 the greatest amount of edema) and without regard to cause. Twenty-eight separate determinations on the twenty-one edematous legs were used in this analysis, the result of which is shown in Table IX. Whether the edema was minimal or maximal, the time of the disappearance of riboflavin was shorter on the average

TABLE IX. EFFECT OF EDEMA ON DISAPPEARANCE OF RIBOFLAVIN

GRADE OF PITTING EDEMA	DETERMINATIONS	50 PER-CENT TIME (MINUTES), MEAN AND S.E.	P*
0 (normal persons)	32	140 ± 6.0	
0 to slight†	12	99 ± 10	<.01
1 to 2	9	91 ± 8	<.01
3 to 4	5	106 ± 4.7	<.01
Swelling without pitting	2	122	

*Significance of difference.

†Patients in this category gave a past history of edema, but no edema was present at time of examination.

than in the normal controls. In all clinical grades of edema, the means of the 50 per-cent times were statistically significantly shorter than the means of the control group.

COMMENT

It is presumed that when riboflavin is injected into interstitial fluid its disappearance is an index of the rate of absorption of the interstitial fluid by both lymphatic and blood capillaries. This rate of absorption in turn is at least partially dependent on the rate of formation of tissue fluid at the arterial end of the capillary bed.

Molecules of riboflavin are small, and thus they can traverse the walls of blood capillaries as easily as they can the walls of lymphatic capillaries. No claim is made that flow of lymph is measured by this method either exclusively or even preferentially.

At the onset it appeared possible that the disappearance of fluorescence of intradermally injected riboflavin might be caused in part by its destruction by the ultraviolet light of the photofluorometer or its diffusion in and dilution with tissue fluid. It was found, however, that ultraviolet light of the magnitude used in the photofluorometer does not destroy riboflavin after four hours of constant exposure and that riboflavin injected into the skin of an amputated leg does not disappear even after many hours. These results indicate that some vital function or functions of living skin are involved in the disappearance of riboflavin from the skin.

The most convincing evidence that disappearance of riboflavin does represent the movement of tissue fluid is the excellent correlation between the findings obtained with the method used in our study and what is already known concerning the physiology of tissue fluid.

A warm environmental temperature and local injections of histamine are known to increase cutaneous blood flow;[1, 5, 10] both these conditions cause a more rapid disappearance of riboflavin.

A cool environmental temperature,[1, 11] dependent position of the legs,[7, 10, 12] occlusion of the venous and lymphatic returns,[7, 12, 14] and local injections of epinephrine are known to decrease peripheral blood flow or lymphatic flow or both; this was reflected by a noticeable delay in disappearance of the riboflavin under such conditions.

Muscular activity increases the flow of blood and lymph from the skin of the active part.[5, 7, 12] In this study, the hastening effect of muscular activity (walking) on the disappearance of riboflavin probably was obscured by the concomitant effect of the dependent position.

The finding that riboflavin disappeared rapidly, on the average, from the skin of edematous extremities must be interpreted with caution in view of the relatively small numbers of patients and normal persons tested. It is possible that the riboflavin was diluted quickly with edema fluid and that its rapid disappearance did not represent rapid transport out of the zones. It was shown, however (Table IX), that the disappearance was equally as rapid whether the edema was minimal or maximal or had disappeared. If dilution were a factor,

it should be more in evidence as the amount of edema increases. Moreover, riboflavin did not disappear from the skin of amputated legs made artificially edematous. The results therefore indicate an increased turnover of fluid in the edematous extremities.

SUMMARY

A method has been described for the photometric measurement of the disappearance of a fluorescent tracer substance (riboflavin) after it has been injected intradermally. The disappearance of the fluorescence may be an index of the rate of flow of cutaneous interstitial fluid.

The disappearance of riboflavin after its injection into the skin of the legs of 20 normal persons was studied under basal conditions. The results were compared with those obtained after subjecting the same persons to various experimental conditions designed to alter the flow of tissue fluid.

An increase in the rate of disappearance of riboflavin, considered to indicate increased flow of tissue fluid, was observed in normal persons in a warm environment and after local injections of histamine.

Slow disappearance of riboflavin, considered to indicate a slower rate of flow of tissue fluid, was observed in normal persons in a cold environment, after local injections of epinephrine, and when the limb in which the injection was made was dependent or obstructed by the inflated cuff of a sphygmomanometer.

No significant alterations in the disappearance of riboflavin were observed in the skin of the lower extremities of patients suffering from chronic occlusive arterial disease.

Disappearance of riboflavin was found to be more rapid in the skin of edematous extremities.

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LABORATORY METHODS

EVALUATION OF A NEW TECHNIQUE FOR LIPASE DETERMINATION

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INTRODUCTION

FOR years there has been considerable interest in the blood lipase level in experimental pancreatitis and various human diseases of the pancreas. Cherry and Crandall[1] and others[2-4] have demonstrated an elevation of blood lipase in dogs following ligation of the pancreatic ducts. Popper and Sorter[5] did not observe these changes. These conflicting results may be explained by the inadequacy of present lipase methods, for they are neither specific nor sensitive.

Seligman[6]*** has devised a colorimetric method for the simultaneous determination of esterase and lipase. A synthetic substrate, β-naphthyl laurate, is hydrolyzed by esterase and lipase to form β-naphthol. Since sodium taurocholate stimulates pancreatic lipase activity and inhibits esterase activity, the two enzymes are determined by comparing the rates of hydrolysis with and without added taurocholate. The β-naphthol resulting from this hydrolysis is coupled with an azo salt to yield a color which may be quantitated colorimetrically. It was the purpose of this study to evaluate the clinical usefulness of this new technique. Studies were made on serum and duodenal contents.

MATERIALS AND METHODS

1. *Blood.*—

a. *Technique:* Determinations of esterase and lipase were made according to Seligman's technique,[6] using 0.2 ml. of fresh serum.

b. *Selection of patients:*

50 normal sera—control
24 sera—cirrhosis of liver
11 sera—infectious hepatitis
3 sera—acute pancreatitis (high amylase levels)

2. *Duodenal contents.*—

a. *Technique:* Because there is much more enzyme in duodenal contents than in serum the original procedure was modified. One milliliter of duodenal juice was diluted to 1/1000. The incubation was reduced from five hours to one hour.

From the Frank Billings Medical Clinic, Department of Medicine, University of Chicago.
This study was facilitated in part by the Institutional Grant to The University of Chicago and partly by the American Cancer Society.
Received for publication, Nov. 10, 1952.
*Fellow of the Belgian American Educational Foundation.
**Aided by a fellowship from the Dazian Foundation for Medical Research.
***Grateful acknowledgment is made to Dr. Herbert Ravin and Dr. Arnold Seligman for their advice and help in starting this project.

b. *Two basal lipase concentrations* were determined in each of 16 normal individuals. (The juice was collected for twenty minutes and divided into two ten-minute specimens.) Five subjects were studied before and after overlapping stimulation by secretin* (1 unit per kilogram intravenously) initially, followed by urecholine (10 mg. subcutaneously) twenty minutes later. Specimens were collected at ten-minute intervals for a total of one hour.

RESULTS

Blood.—Results are expressed in μg of β-naphthol rather than Seligman units to avoid confusion, since the hours of incubation are part of the definition of this unit and we have found it expedient to vary the incubation period from one type of body fluid to another. Esterase values are expressed in μg of β-naphthol liberated from β-naphthyl laurate after five hours' incubation at 37.5° C. with 0.2 ml. of fresh serum. Lipase, had it been present, would have been expressed as μg of β-naphthol in excess of those liberated by esterase if sodium taurocholate was added to the above system.

a. *Normal sera:* In 50 sera an average esterase activity of 53 μg was found, ranging from 36 to 78 μg. Taurocholate actually induced an inhibition varying from 10 to 42 per cent, thus lipase was not detected.

b. *Cirrhosis of liver* (Table I): Among 24 cases studied, 20 had values lower than the normal range and 4 exceeded 36 μg. The mean value for esterase was 27.4 μg. Taurocholate inhibition was similar to that noted

TABLE I. SERUM ESTERASE IN CIRRHOSIS OF LIVER

CASE	ESTERASE (μg)	PROT.	ALB. (GRAM %)	GLOB.	COMMENT
1	9.0	7.55	3.36	4.19	Icterus, ascites, edema
2	10.0	5.84	2.51	3.33	Icterus, ascites
3	14.0	5.93	3.49	2.44	Icterus, ascites
4	15.3	5.60	2.10	3.50	Banti's syndrome?
5	16.8	6.59	2.13	3.66	Icterus
6	17.0	7.70	3.94	3.74	Terminal stage of biliary cirrhosis
7	20.0	6.36	2.94	3.42	Ascites
8	20.4	6.94	2.71	4.28	Icterus, ascites
9	20.5	5.68	2.29	3.39	Icterus
10	22.6	6.74	2.79	3.95	Icterus
11	22.7	8.74	3.51	5.23	Banti's syndrome
12	24.0	7.29	4.45	2.84	
13	26.0	5.62	2.98	2.68	Ascites
14	26.4	6.74	3.13	3.61	Banti's syndrome, hematemesis, liver failure
15	27.0	5.16	2.33	1.81	Hematemesis
16	28.0	7.00	2.80	4.20	Terminal stage of biliary cirrhosis
17	30.4	10.92	3.37	6.55	Ascites
18	30.6	6.94	3.28	2.66	Icterus
19	32.0	6.72	3.57	3.15	Regional enteritis
20	32.0	9.07	4.45	4.62	Icterus, ulcerative colitis
21	40.0	7.23	4.69	2 59	
22	42.5	7.30	4.10	3.20	
23	46.6	7.98	4.02	3.96	Esophageal varices
24	63.5	7.02	4.71	2.30	Chronic alcoholism, fatty degeneration of liver

Esterase results are expressed as μg of β-naphthol liberated after five hours' incubation with 0.2 ml. of fresh serum.

*Secretin was generously supplied by the Lilly Research Laboratories.

TABLE II. CORRELATION BETWEEN ESTERASE AND SERUM ALBUMIN

ALBUMIN (GM. %)	ESTERASE (μG) MIN.	MAX.	MEAN	NUMBER OF CASES
Less than 3 grams	10	28	19.4	10
3 to 4 grams	9	48.8	30.3	14
Over 4 grams	24	72	43.6	12

Esterase results are expressed as μg of β-naphthol liberated after 5 hours' incubation with 0.2 ml. of fresh serum.

in normal sera. The majority of the cases studied had liver failure with concomitant ascites, jaundice, or hematemesis. The results indicate a correlation between the total esterase activity and the serum albumin level (Table II), as previously demonstrated by Faber,[6] Lagerlöf,[7] and Goldner.[8]

c. Among 11 cases of infectious hepatitis (Table III) some decreased esterase activity was found in five patients. The mean value was 34 μg (lower range of normal). It should be emphasized that low values were found only in those cases complicated by other chronic disease (neoplasm, ulcerative colitis). Lagerlöf[9] found no decrease in esterase activity in infectious hepatitis.

TABLE III. INFECTIOUS HEPATITIS

CASE	ESTERASE (μG)	PROT.	ALB. (GRAM %)	GLOB.	OTHER PATHOLOGY
1	19.5				Neuroblastoma
2	23.0	5.95	3.22	2.73	Cardiac failure
3	27.5	5.27	2.92	2.80	Ulcerative colitis
4	29.5	6.20	3.51	5.23	Infectious mononucleosis
5	30.8	7.42	3.98	3.44	
6	30.9	6.29	3.51	2.78	Ulcerative colitis; colonic cancer
7	37.5	6.44	3.91	2.53	
8	40.2	6.92	4.21	2.70	
9	43.0	5.92	4.25	1.67	
10	45.2				
11	48.8	6.92	3.88	3.04	

Esterase results are expressed as μg of β-naphthol liberated after five hours' incubation with 0.2 ml. of fresh serum.

d. In 7 instances (Table IV) an attempt was made to determine the contribution of cholinesterase to "total esterase" as determined above. For this purpose physostigmine salicylate (4 μg per milliliter) was added to the system previously described. The inhibition obtained after five

TABLE IV. PHYSOSTIGMINE SALICYLATE (4 μg/ML.) ESTERASE ACTIVITY (μg)

WITHOUT	WITH	% INHIBITION
22.5	15.0	33
25.0	15.9	36
29.4	17.6	40
36.7	25.4	30
41.7	24.3	42
37.0	19.7	47
44.5	27.0	39

Esterase results are expressed as μg of β-naphthol liberated after five hours' incubation with 0.2 ml. of fresh serum.

hours of incubation ranged from 30 to 47 per cent. In four experiments physostigmine salicylate and taurocholate were added simultaneously, increasing the inhibition to about 60 per cent.

e. In 3 cases of acute pancreatitis (amylase activity of 480, 2000 and 3000 units) we were unable to find any lipase in blood. Daily urinary lipase determinations performed in one case were not elevated.

Duodenal drainage.—Esterase values are expressed as milligrams of β-naphthol liberated from β-naphthyl laurate after one hour's incubation at 37.5° C. with 0.2 ml. of fresh duodenal contents. Lipase values are expressed as milligrams of β-naphthol in excess of those liberated by esterase if sodium taurocholate was added to the above system. In our assays of the duodenal contents the addition of sodium taurocholate produced an average increase of almost sixfold in the hydrolysis of β-naphthyl laurate. In 16 cases the lipase activity (without stimulants) varied from 2.60 mg. to 13.50 mg. Although the lipase activity varied over a wide range from one patient to another, the activity in the same individual was fairly constant. In five normal persons the injection of secretin induced the usual increase in the volume of duodenal drainage; lipase concentration decreased. In contrast urecholine produced a definite increase in enzyme concentration (Table V). Urecholine did not alter the volume response to secretin, although the total output of enzyme was consistently higher.

TABLE V. EFFECT OF SECRETIN AND URECHOLINE ON LIPASE CONCENTRATION OF DUODENAL CONTENTS

CASE		MINUTES					
		0-10	10-20	20-30	30-40	40-50	50-60
1	Volume	15	13	33	29	19	20
	Lipase concentration (mg.)	4.46	5.50	3.68	3.32	4.42	7.06
2	Volume	12	10	30	26	24	22
	Lipase concentration (mg.)	5.08	4.92	0.98	1.12	3.46	5.52
3	Volume	5	5	15	20	20	22
	Lipase concentration (mg.)	7.48	6.36	3.18	3.12	8.30	8.85
4	Volume	17	19	41	48	46	42
	Lipase concentration (mg.)	9.16	9.72	6.48	6.96	13.08	15.38
5	Volume	5	6	15	20		
	Lipase concentration (mg.)	6.12	5.28	2.28	2.72		

SECRETIN I.V. — URECHOLINE I.M.

Results are expressed as milligrams of β-naphthol liberated after one hour's incubation with 0.2 ml. of fresh duodenal contents.

DISCUSSION

In this series of cases, Seligman's technique showed no serum lipase activity, even in three cases of acute pancreatitis with elevated amylase activity. Seligman[7] found lipase in only five of thirteen cases of acute pancreatitis. There was no correlation with serum amylase. Seligman has reported similar low lipase values in the blood of eight patients with common duct obstruction secondary to causes other than pancreatic pathology; in cancer of the pancreas he demonstrated lipase in one of six cases. Using olive oil as a substrate, Comfort[8] noted an elevation of lipase in 99 per cent of patients with acute pancreatitis.

The blood lipase data suggest that this technique is not applicable to the diagnosis of acute and chronic pancreatic disease. It has been shown that human serum is very rich in esterase. Patients with cirrhosis and infectious hepatitis accompanied by low serum albumin show a similar decrease in the hydrolytic activity of their serum on β-naphthyl laurate. This suggests that this technique reflects liver esterase activity (pseudocholinesterase and ordinary esterase). Faber,[9] using a gasometric technique, and Goldner,[10] using Gomori's,[11*] colorimetric determination, noticed the same decrease in esterase activity, and the same correlation with serum albumin. This hypothesis is further substantiated by the fact that physostigmine salicylate, which inhibits cholinesterase specifically, induced a decrease of activity in the sera of seven patients.

Since the addition of taurocholate increased this inhibition from 40 to 60 per cent (approximately), we may state that a significant part of this hydrolytic activity is due to esterase rather than pancreatic lipase. Since taurocholate activates pancreatic lipase and inhibits esterase, the lack of lipase in normal blood serum might be explained by a predominance of esterase inhibition over lipase activation. Thus these techniques do not exclude the existence of significant amounts of lipase in blood. Further investigation is required to settle this point. Serial determinations of urinary lipase in one case of acute pancreatitis showed no increase, suggesting that a low renal threshold for lipase is not the explanation for the absence of lipase in blood.

The basal values of lipase activity vary from one patient to another over a wide range, but in the same patient they are constant. The results obtained with Seligman's technique confirm those of others,[12, 13] namely: decrease in enzyme concentration after secretin followed by an increase after urecholine. Both secretin and urecholine produced increased enzyme output per unit time. Seligman's lipase method is more sensitive and yet less time consuming than existing procedures; it may prove to be a valuable adjunct in the study of pancreatic enzymes.

SUMMARY

The clinical usefulness of Seligman's new technique of lipase and esterase determination in the diagnosis of pancreatic disease was evaluated. Blood lipase determinations by this procedure during acute pancreatitis were of no value in our series. Various possible mechanisms are discussed to explain the absence of blood lipase by our determinations. Study of lipase behavior in duodenal juice, using secretin and urecholine as pancreatic stimulants, suggest a possible clinical use for this new technique.

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*The authors are grateful to Dr. George Gomori for his many helpful suggestions.

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THE PHOTOMETRIC MICRODETERMINATION OF GAMMA GLOBULIN IN CEREBROSPINAL FLUID BY A QUANTITATIVE PROTEIN FLOCCULATION-NINHYDRIN REACTION*

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IT HAS long been recognized that in many neurological conditions, e.g., multiple sclerosis, changes may occur in the globulin fractions without significant increases in the total protein values.[1, 2] Many qualitative tests,[3, 4] as well as quantitative methods employing salt fractionation,[5-9] have been used to determine increased amounts of total globulins in the cerebrospinal fluid. The relatively large amounts of cerebrospinal fluid required, as well as the involved and laborious analytical methods used, have made cerebrospinal fluid albumin/globulin ratio determinations impractical for most clinical laboratories. In addition, the application of salt fractionation methods to the low protein concentrations present in spinal fluids has been criticized both by Lange[10] and Schied and Schied[11, 12] as being highly inaccurate.

Electrophoretic methods for the separation and quantitative determination of the various protein fractions present in concentrated samples of cerebrospinal fluid have been reported by several groups of investigators including Kabat and associates[13] and Schied and Schied.[11] These investigators found the same protein components present in the cerebrospinal fluid as are found in blood plasma. They also found that the major changes in disease cases occur in the albumin, beta globulin, and gamma globulin fractions of the cerebrospinal fluid. The increase in the albumin fraction in disease cases follows closely the total protein value, whereas the increase in the other two fractions is more specific and may indicate an antibody response.[13] The electrophoretic method, too, is impractical for routine clinical work as it requires expensive equipment, skilled technical help, large volumes of spinal fluid, and permits only a few determinations to be performed daily.

Immunochemical techniques[14] have been proposed for the quantitative microdeterminations of specific protein fractions, e.g., albumin and gamma globulin, in cerebrospinal fluid by Kabat and his co-workers,[15] and were employed by them as a diagnostic test for cases of multiple sclerosis.[16] According to Merritt,[17] immunochemical procedures are excluded from most clinical laboratories because of the difficulties involved in the preparation and assay of the antisera used and the intricate and time-consuming analytical techniques required.

It is the experimental difficulties encountered with the aforementioned procedures which account for the widespread use of a number of qualitative[12, 18, 19]

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Received for publication, Feb. 12, 1953.
*Aided by a grant from the National Multiple Sclerosis Society, New York, N. Y.

and semiquantitative[20] protein flocculation reactions for the detection of protein changes in pathologic cerebrospinal fluid. The physicochemical basis underlying these protein flocculation reactions and their relationships to the various electrophoretic protein fractions present in both serum and cerebrospinal fluid has been discussed recently in a review paper by one of us.[21] These principles were used to convert a previously published method[22] for the semiquantitative determination of increased gamma globulin in cerebrospinal fluid into a *quantitative* photometric micro-procedure for serum gamma globulin[23] which checks closely with the electrophoretic method.[24]

The present paper deals with the application of this quantitative protein flocculation-ninhydrin method to the photometric, micro-determination of the gamma globulin content of cerebrospinal fluid. Unlike the previously published procedure it employs a *stable* desoxycholic acid-cholesterol emulsion[25] which gives more reproducible results with the smaller amounts of gamma globulin present in cerebrospinal fluid. It requires only 1 ml. of spinal fluid per determination and recovers added gamma globulin with an accuracy of ± 5 per cent. It uses only readily available equipment and reagents and permits the performance of large numbers of determinations daily. It is therefore a procedure which is simple enough to be performed in most clinical laboratories yet checks closely with the immunochemical method over a wide range of cerebrospinal fluid gamma globulin values.

METHODS AND MATERIALS

A. *Reagents and Apparatus:*

1. Sodium chloride, 0.85 per cent solution.

2. Sodium chloride, 11.0 per cent solution.

3. Phosphate buffer (pH 7.00)–0.4M. Dissolve 39.2 Gm. anhydrous Na_2HPO_4 and 20.5 Gm. KH_2PO_4 in a liter of solution. Adjust pH to 7.00 ± 0.02 with a pH meter.

4. Desoxycholic acid-cholesterol (D.C.A.-Chol.) emulsion.

a. Stock solution. Dissolve 0.125 Gm. of C.P. desoxycholic acid (Fisher Scientific Co.) and 0.125 Gm. of C.P. cholesterol (Fisher Scientific Co.) in 100 ml. of absolute alcohol warming the mixture on a hot plate, if necessary, to completely dissolve the components. This solution if stoppered tightly and stored in a refrigerator can be kept indefinitely.

b. Preparation of stable emulsion. Heat 35 volumes of N/70 NaOH in an Erlenmeyer flask to 65° C. Add 2 volumes of the alcoholic D.C.A.-Chol. solution drop by drop with constant agitation until a homogeneous emulsion is formed. Continue heating the mixture at a temperature just below the boiling point until only 30 volumes of the emulsion remain. Cool to room temperature, transfer to a tightly stoppered bottle and store in an incubator at 37° C. when not in use. This emulsion is stable over a period of several months.

5. Gamma globulin standard solution. Weigh out approximately 20 mg. of electrophoretically pure gamma globulin (see reference[14], p. 461 for preparation) into a wide-mouthed 125 ml. glass bottle. Add 50 ml. of 0.85 per cent NaCl under vacuum conditions, slowly and with constant agitation until most of the protein goes into solution. Centrifuge at 3,000 r.p.m. to remove any insoluble material and transfer the clear supernatant fluid to a glass-stoppered bottle. Determine the exact protein content of this solution with a micro-Kjeldehl digestion and distillation method and dilute with 0.85 per cent NaCl to a concentration of about 200 μg per milliliter. A small crystal of thymol is added as a preservative and the solution stored in a refrigerator when not in use. This solution remains stable over a period of several months.

6. Hayem's solution. Dissolve 10.0 Gm. of NaCl, 11.0 Gm. Na_2SO_4 (anhydrous) and 2.5 Gm. $HgCl_2$ in a liter of solution.

7. Ether. Reagent grade, anhydrous.

8. Sodium hydroxide solution, 0.1N.

9. Ninhydrin reagent. The ninhydrin (obtained from Dougherty Chemical Co., Jamaica, N. Y.) used in these experiments was twice recrystallized as described by Moore and Stein.[26]

a. Citrate buffer (pH 5.0)–0.2M. Dissolve 21.008 Gm. citric acid (reagent grade), and 200 ml. of 1N NaOH in 500 ml. of solution in a volumetric flask. Add a small crystal of thymol as a preservative against mold growth.

b. Ethylene glycol monomethyl ether (Matheson Co., East Rutherford, N. J.). Purify the commercial product by distilling under vacuum.

c. Ninhydrin solution. Dissolve 68 mg. of anhydrous stannous chloride (Flaked Stannochlor obtained from Metal and Thermit Corporation, New York, N. Y.) in 50 ml. of citrate buffer in a 100 ml. glass-stoppered graduated cylinder. Add to this solution 2.0 Gm. of recrystallized ninhydrin and then 50 ml. of the purified monomethyl ether. Stopper and dissolve by shaking until all the solids are in solution. A play of colors occurs during the solution process, but the final reagent has a yellow color and is water clear. If the solution remains turbid it can usually be clarified by placing the graduate in warm water. A sufficient amount of this reagent is prepared for each day's run as it deteriorates on longer standing.

10. Diluent solution. Mix equal volumes of water and n-propanol (reagent grade).

11. Cellophane tubing. 1 inch wide. (Visking Corp., Chicago, Ill.)

12. Pan, Aluminum. 12 inches by 18 inches by 4 inches deep, for boiling water bath.

13. Spectrophotometer. Beckman Model D.U. with test tube adapter or other similar instrument adaptable for reading small test tubes.

14. Syringe pipettes, Cornwall, 2 ml. capacity. Obtainable from Becton, Dickinson and Co., Rutherford, N. J.

15. Cuvettes, test tube, selected. Select 75 by 12 mm. outer diameter test tubes, Pyrex, without lip, for light absorption characteristics in a manner similar to that described in the paper by Moore and Stein.[26]

16. Test tubes, 5 ml. capacity, graduated in 0.1 ml. divisions.

Procedure.—Prepare a Cellophane bag for each cerebrospinal fluid sample from 3 to 4 inch lengths of Cellophane tubing. Pipette into the Cellophane bag an accurately measured amount, i.e., 1.5 to 3.5 ml., depending upon size of the available sample of the cerebrospinal fluid and tie off the open end with a double knot leaving only a small amount of air space. Label the bag by stapling on tags above the knotted end and place the bags into a large sized filter flask filled with cold tap water for three to four hours. Cold tap water is then run through the flask, by means of a piece of rubber tubing extending to the bottom, for twelve hours or longer. It was found experimentally that by performing the dialysis step in this manner, the volume of the cerebrospinal fluid could be markedly reduced.

After dialysis, the cerebrospinal fluid was transferred quantitatively to a 5 ml. graduated test tube, containing 0.08 ml. of 11 per cent NaCl per milliliter of original cerebrospinal fluid, by washing the bag with distilled water until the original volume of the spinal fluid is restored and the contents mixed by inversion.

Pipette, in triplicate, 1.00 ml. aliquots of each sample into the selected Pyrex test tubes. For each run, pipette in quadruplicate, 0.00 ml. (blanks), 0.5 ml. and 1.0 ml. of standard gamma globulin solution, adding the proper amount of 0.85 per cent NaCl solution to each tube so that the total volume is exactly 1.00 ml. By means of syringe pipettes, add 0.5 ml. phosphate buffer, 0.75 ml. of Hayem's solution, and 0.75 ml. of the D.C.A.-Chol. emulsion to each tube and mix the contents by inverting each tube separately.

Place the Kahn racks containing the tubes in the 37.5° C. water bath for forty-five minutes. Remove tubes from water bath and centrifuge for fifteen minutes at 3,000 r.p.m. using multiple carriers. Pour off supernatant fluid and invert the tubes in the racks, allowing any remaining fluid to drain on to cotton gauze placed on the bottom of the racks. Break up the precipitate by tapping the bottoms and add approximately 2 ml. of ether to each tube using a 20 ml. glass syringe. Mix well to wash precipitate and centrifuge again at 3,000 r.p.m. for five minutes. Aspirate off the ether layer with gentle suction using either a glass

TABLE I. COMPARISON OF QUANTITATIVE PROTEIN FLOCCULATION-NINHYDRIN AND IMMUNOCHEMICAL METHODS FOR CEREBROSPINAL FLUIDS FROM NEUROLOGICAL CASES

C.S.F. NO.	TOTAL PROTEIN (MG./100 ML.)	PROTEIN FLOCCULATION-NINHYDRIN GAMMA GLOBULIN (MG./100 ML.)	IMMUNOCHEMICAL GAMMA GLOBULIN (MG./100 ML.)	DIFFERENCE (MG./100 ML.)	GAMMA GLOB. / T.P. PER CENT
1	83	14.2; 14.4; 14.0 Average = 14.2	14.9	−0.7	18.0
2	85	0.81; 0.83; 0.90 Average = 0.85	0.7	+0.2	0.8
3	54	4.1; 4.1; 4.0 Average = 4.1	4.1	±0.0	7.6
4	49	5.0; 4.8; 4.5 Average = 4.8	4.3	+0.5	8.8
5	62	8.0; 8.4; 8.0 Average = 8.1	8.3	−0.2	13.4
6	46	5.0; 5.4; 5.6 Average = 5.3	4.6	+0.7	10.0
7	34	1.4; 1.7; 1.5 Average = 1.5	1.8	−0.3	5.3
8	68	6.2; 6.1; 6.2 Average = 6.2	7.0	−0.8	10.3
9	42	10.2; 9.5; 9.7 Average = 9.8	9.6	+0.2	22.9
10	60	12.8; 12.9; 12.6 Average = 12.8	12.4	+0.4	20.7
11	60	3.9; 3.7; 3.9 Average = 3.8	3.6	+0.2	6.0
12	38	5.3; 5.5; 5.7 Average = 5.5	5.4	+0.1	14.2
13	50	7.9; 7.5; 7.2 Average = 7.5	8.1	−0.6	16.2
14	74	5.7; 5.8; 5.9 Average = 5.8	5.3	+0.5	7.2
15	43	4.3; 4.0; 4.2 Average = 4.2	3.9	+0.3	9.1

The standard deviation of the flocculation-ninhydrin method as calculated from the replicate determinations according to Mainland[31] is ±.18 mg./100 ml. The accepted normal range for this method is the same as that proposed by Kabat and co-workers,[16] i.e., 2-6 mg. of gamma globulin per 100 ml. of cerebrospinal fluid and gamma globulin/total protein ratio values from 5.7 to 13.0.

pipette or a large stainless needle with about a 1/16 inch opening. Repeat the washing, centrifugation, and aspiration once more. Remove all traces of ether by placing racks with tubes in the 37.5° C. water bath for an hour or longer.

Add to each tube 1.00 ml. of 0.85 per cent NaCl and 0.2 ml. of 0.1N NaOH and mix by shaking the racks together with the tubes. Add 0.5 ml. of freshly prepared ninhydrin reagent to each tube and mix each tube separately by tapping with the fingers. Place Kahn racks with the tubes in a vigorously boiling water bath for thirty minutes. Mix the contents of the tubes at least twice during the heating period so as to insure maximum color development. Allow to cool to room temperature and then add 1.5 ml. of the n-propanol-water diluent to each tube. Mix contents and centrifuge tubes at 3,000 r.p.m. for five minutes to bring

down any insoluble material. Clean outside of each tube well and measure the optical density values in a spectrophotometer at 570 millimicrons directly in the original test tubes as against pure diluent solution as a blank. Subtract the average blank value from both standards and unknowns to obtain the corrected optical density values.

Calculations.—

$$\text{Gamma globulin} \left\{\begin{matrix}\text{mg. per 100 ml.} \\ \text{cerebrospinal fluid}\end{matrix}\right\} = \frac{\text{O.D. unk.}}{\text{O.D. stand.}} \times \frac{\text{Gamma globulin standard (mg.)}}{} \times \frac{100}{\text{ml. of C.S.F. used}}$$

For a gamma globulin standard solution such that 1 ml. = 0.20 mg. and a 1.00 ml. of cerebrospinal fluid sample, the equation becomes:

$$\text{Gamma globulin} \left\{\begin{matrix}\text{mg. per 100 ml.} \\ \text{cerebrospinal fluid}\end{matrix}\right\} = \frac{\text{O.D. unk.}}{\text{O.D. stand.}} \times 20.$$

RESULTS

Comparison of Quantitative Protein Flocculation-Ninhydrin Method With Immunochemical Method.—The gamma globulin content of 100 cerebrospinal fluids from patients with various neurological diseases were analyzed by the immunochemical method of Kabat and associates[15] and the results compared with those obtained, on the same fluids, with the quantitative protein flocculation-ninhydrin method described above. Some typical sets of results are presented in Table I to show the reproducibility of the method, its comparability to the immunochemical method, and the clinical importance of the total protein/gamma globulin ratio.

TABLE II. RECOVERY AND DILUTION RUNS FOR GAMMA GLOBULIN IN CEREBROSPINAL FLUID WITH PHOTOMETRIC PROTEIN FLOCCULATION-NINHYDRIN METHOD

DILUTION RUNS

GAMMA GLOBULIN IN ORIGINAL SAMPLE (MG./100 ML.)	DILUTION	GAMMA GLOBULIN CALCULATED (MG./100 ML.)	GAMMA GLOBULIN FOUND (MG./100 ML.)	DIFFERENCE (MG./100 ML.)
35.1	1:1.33	26.4	28.4	+2.0
T.P. = 256	1:2	17.5	16.1	−1.4
G.G./T.P. = 13.7%				
20.0	1:2	10.0	10.1	+0.1
T.P. = 208	1:4	5.0	6.0	+1.0
G.G./T.P. = 9.6%				
27.6	1:1.33	20.7	20.0	−0.7
T.P. = 309	1:2	13.8	13.5	−0.3
G.G./T.P. = 9.0%	1:4	6.9	8.0	+1.1
26.0	1:1.33	19.5	19.3	−0.2
T.P. = 234	1:2	13.0	14.1	+1.1
G.G./T.P. = 11.1%	1:4	6.5	7.2	+0.7

RECOVERY RUNS

GAMMA GLOBULIN IN ORIGINAL SAMPLE (MG./100 ML.)	GAMMA GLOBULIN ADDED (MG./100 ML.)	GAMMA GLOBULIN CALCULATED (MG./100 ML.)	GAMMA GLOBULIN FOUND (MG./100 ML.)	DIFFERENCE (MG./100 ML.)
4.3	10.0	14.3	14.1	−0.2
2.1	10.0	12.1	11.5	−0.6
4.2	4.4	8.6	9.3	+0.7
7.2	4.4	11.6	11.6	±0.0
4.0	4.4	8.4	8.3	−0.1
2.0	4.4	6.4	6.7	+0.3

The data shown in Table II furnish additional experimental support regarding the accuracy of the protein flocculation-ninhydrin method for gamma globulin by showing the recoveries obtained when small amounts of pure gamma globulin are added to cerebrospinal fluid and the values obtained when fluids of high gamma globulin content are diluted to successively lower values.

Table III presents a summary of the cerebrospinal fluid data obtained for the entire series of 100 neurological cases with the two procedures. This table shows the distribution of the gamma globulin values obtained for these randomly selected cases and the close correlation of the mean values of the results obtained by the immunochemical and protein flocculation-ninhydrin methods over a wide range of gamma globulin values.

TABLE III. DISTRIBUTION OF GAMMA GLOBULIN VALUES FOR 100 CEREBROSPINAL FLUIDS* FROM NEUROLOGICAL CASES AND COMPARISON OF RESULTS OBTAINED WITH THE IMMUNOCHEMICAL AND PROTEIN FLOCCULATION-NINHYDRIN METHODS

	GAMMA GLOBULIN—MILLIGRAMS PER 100 ML.					
	0-1.0	1.1-2.0	2.1-3.0	3.1-4.0	4.1-5.0	5.1-6.0
No. of cases	1	3	12	8	18	9
Flocculation-ninhydrin (Mean)	0.7	1.7	2.6	3.5	4.5	5.7
Immunochemical (Mean)	1.1	2.4	2.5	3.1	4.1	5.4
	GAMMA GLOBULIN—MILLIGRAMS PER 100 ML.					
	6.1-7.0	7.1-8.0	8.1-9.0	9.1-10.0	10.1-12.0	12.0-20.0
No. of cases	18	6	5	4	12	4
Flocculation-ninhydrin (Mean)	6.5	7.5	8.4	9.2	11.1	14.8
Immunochemical (Mean)	6.2	7.3	8.4	8.6	11.4	14.1

The total protein values for this series as determined by the Denis and Ayer[33] method ranges from 21 to 118 mg. per 100 ml. of spinal fluid.

*These cerebrospinal fluids were obtained through the courtesy of Dr. E. A. Kabat and Miss Ada E. Bezer, Department of Bacteriology, College of Physicians and Surgeons, Columbia University Medical School, New York, N. Y. The total protein and immunochemical gamma globulin determinations were performed in Dr. Kabat's Laboratory.

DISCUSSION

The various factors which influence the quantitative flocculation of gamma globulin from solution have been discussed in detail in a previous publication.[23] The basic principle of the method involves the interaction of a particular protein fraction, e.g., gamma globulin, with a lipid emulsion in the presence of Hayem's solution to form a precipitate whose protein content may be determined, after ether washing, photometrically with the ninhydrin reaction.[26] That such protein-lipid interactions may have a high order of specificity for a particular protein fraction has been suggested in a review paper by Grabar.[27] A similar procedure involving the interaction between hematin and human serum albumin has been published recently by Rosenfeld and Surgenor[28] and found to be highly selective for this protein fraction even in the presence of the other components of plasma.

The major change from the method as used for serum gamma globulins is the introduction of a new stable emulsion composed of a mixture of desoxycholic acid and cholesterol. This preparation is similar to that proposed by Steinberg[25] and by Pollak,[29] and found to remain stable by these investigators over long periods of time. We have found this type of emulsion to have a number of

important advantages over the cephalin-cholesterol emulsion of Hanger[30] previously used. Both desoxycholic acid and cholesterol are chemically pure substances which dissolve readily in alcohol to give a stable solution. The emulsion prepared from this alcoholic solution contains no amino groups which react with ninhydrin, gives more cohesive precipitates than the cephalin-cholesterol emulsion, and gives colors which follow the Bouger-Lambert-Beer Law over the range of gamma globulin values usually found in cerebrospinal fluids, i.e., from 25 μg to 400 μg. The same emulsion may also be used for serum gamma globulin determinations with increased reproducibility of replicate values.

The excellent reproducibility of the modified method for cerebrospinal fluid gamma globulin is shown by the data, as given in Table I, for 15 cases run in triplicate. The standard deviation (S.D.), calculated according to Mainland,[31] is ± 0.18 mg. per 100 ml. of spinal fluid for this group of cases. The standard error of the mean is ± 0.10 mg. per 100 ml. of spinal fluid for a sample run in triplicate, and therefore differences between samples greater than ± 0.3 mg. of gamma globulin per 100 ml. of spinal fluid would be significant at the 1 per cent level. Since no comparable analytical data have been published for the immunochemical method by Kabat and associates,[15, 16] the results obtained with the protein flocculation-ninhydrin method are therefore expressed simply as difference values from those obtained with the immunochemical method in Tables I and II. It can be seen from the data in these tables that the protein flocculation-ninhydrin method checks with the immunochemical method with an average value of ± 0.5 mg. of gamma globulin per 100 ml. of spinal fluid, and that the errors are most probably experimental in that there is an almost equal distribution of positive and negative differences among the results.

Further experimental evidence for the validity of the method is shown by the excellent recoveries of added gamma globulin and the good results obtained by the dilution experiments, with high gamma globulin-spinal fluids, as given in Table II.

The distribution of the gamma globulin values obtained for the 100 randomly selected cerebrospinal fluids with various neurological disorders (including cases under observation which are presumed normal) shows four distinct peaks as is illustrated in Table III. These peak mean values for gamma globulin obtained with the protein flocculation-ninhydrin method are obtained at the following levels: 2.6 (12 cases); 4.5 (18 cases); 6.5 (18 cases) and 11.1 (12 cases)—all expressed as milligrams per 100 ml. of spinal fluid. The 2.6 and 4.5 values obtained check closely with the mean values of 2.9 (T.P. = 19-39) and 4.5 (T.P. = 40-54) reported by Kabat and his co-workers[16] for their presumed *normal* spinal fluids at the two given total protein ranges. The third peak value of 6.5 corresponds to the approximate mean value reported for multiple sclerosis and other related diseases by Kabat and collaborators,[15] whereas the fourth peak value of 11.1 corresponds rather well with the range of values reported for cases of neurosyphilis by these investigators.

As shown by Kabat and co-workers,[13] when the total proteins exceed 100 mg. per 100 ml. of spinal fluid the gamma globulin values are almost always above

normal limits and the G.G./T.P. ratio (as seen in Table II) is usually in the normal range. On the other hand, the clinical importance of the G.G./T.P. ratio value is shown by the data in Table I where 6 of the 15 cases with slightly increased total protein values show a G.G./T.P. ratio above 13.0 per cent, the upper limit of normal reported by Kabat and associates.[16]

It is now generally believed that the antibody molecule is a "modified" globulin, usually a gamma globulin, and that it may be formed in any cell capable of normal protein synthesis.[32] The presence of an increased cerebrospinal fluid gamma globulin value with a normal serum gamma globulin has led Kabat and his co-workers[13, 16] to the conclusion that a portion of the cerebrospinal fluid globulin is synthesized within the central nervous system. This concept illustrates the importance of simultaneous serum and cerebrospinal fluid gamma globulin determinations in diseases of the central nervous system, and such studies are now being carried out in this hospital for such diseases as multiple sclerosis, postencephalitic Parkinsonism, and other related neurological disorders. In addition the principles of the protein flocculation-ninnydrin method are being extended to the quantitative microdetermination of the other protein fractions present in cerebrospinal fluid, e.g., albumin, beta globulins, etc.

SUMMARY

A previously published protein flocculation-ninhydrin method for serum gamma globulin has been applied to the photometric quantitative microdetermination of gamma globulin in dialyzed cerebrospinal fluid.

The method previously described was modified by the use of a stable emulsion of desoxycholic acid-cholesterol to flocculate the gamma globulin quantitatively from the dialyzed spinal fluid under rigidly controlled experimental conditions. This new emulsion gives highly reproducible results, and extends the useful range of the method to the lower gamma globulin levels found in spinal fluid.

The method has a S.D. of ± 0.18 mg. per 100 ml. of spinal fluid and checks with the immunochemical method to ± 0.5 mg. per 100 ml. C.S.F. over a wide range of gamma globulin values (0.7-16.9 mg. per 100 ml.). Good recoveries (± 5 per cent, average) are obtained either by dilution of high gamma globulin-containing C.S.F. or upon addition of pure gamma globulin solution.

Analysis of the gamma globulin content (milligrams per 100 ml. C.S.F.) of 100 spinal fluids from randomly selected neurological cases shows peak mean values at 2.6 (12 cases) and 4.5 (18 cases) in the normal range; and 6.5 (18 cases) and 11.1 (12 cases) in the abnormal range. The latter two values correspond to those found for multiple sclerosis and neurosyphilis cases, respectively, whose total protein (T.P.) values are below 100 mg. per 100 ml. C.S.F.

Although changes in the absolute gamma globulin level are usually the best diagnostic aid in neurological diseases, the G.G./T.P. ratio, when increased provides additional useful clinical information especially when the gamma globulin is less than 6 mg. per 100 ml. C.S.F.

Since it is now believed that antibodies, e.g., spinal fluid gamma globulin, may be synthesized directly in the cells of the central nervous system in neurological disorders, the simultaneous determination of serum and cerebrospinal fluid gamma globulins has become increasingly important and is being studied in this laboratory for such diseases as multiple sclerosis, Parkinsonism, poliomyelitis, etc.

Acknowledgment.—The authors wish to acknowledge the aid and encouragement of Dr. Bruno W. Volk, Director of Laboratories, who approved this study under a grant from the National Multiple Sclerosis Society. We also wish to express our appreciation to Dr. Joseph Steigman, Department of Analytical Chemistry of The Polytechnic Institute of Brooklyn for his criticism of the analytical data in this paper and to Miss Renee Eisner for the typing and editing of the manuscript.

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UNSTAINED CELL COUNTS AS A METHOD OF EVALUATING CANCEROCIDAL AGENTS

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CONCOMITANT with the apparently increasing incidence of cancer in man, there has been an ever-increasing attempt to effect complete cures through surgery and the use of chemical and physical agents. Already there is a formidable list of chemical and physical agents[1] which have been evaluated for cancerocidal potentialities and which, in almost all instances, have been found to be completely ineffective. Without doubt this list of agents will continue to grow, and considerable money and much time and effort will be spent in their evaluation.

Various procedures for screening and evaluating cancerocidal agents have been employed. The screening methods now used include in vivo and in vitro techniques.

In the in vivo methods, the test object usually consists of one of the following: transplantable solid tumors, transplantable intraperitoneal tumors, transplantable leukemia, spontaneous tumors, or induced tumors. The test material is administered at the time of tumor inoculation, at the first sign of tumor growth, or after the growth is well established. The test agent in single or multiple doses is given orally, intravenously, subcutaneously, intraperitoneally, or into and around the tumor. The criteria used for measuring the effect of the agent is the percentage of tumor takes, measurements of tumor growth or regression, histologic observations, time of survival, or subsequent transplantability of the tumor.

The in vitro methods study the effect of the agent on tumor tissue grown in tissue culture, or on the membranes of developing chicks or the effect of the reagent on the transplantability or the metabolism of the tumor tissue.

Karnofsky,[2] who has reviewed current screening methods, concludes "one of the important fields in cancer research is the development of more precise methods for testing substances in the laboratory for chemotherapeutic activity."

The purpose of these experiments is to test the "method of unstained cell counts" as a screening procedure for cancerocidal agents.

The Method of Unstained Cell Counts.—Following the introduction of trypan blue and other vital stains into common use shortly after 1900, it was repeatedly observed that injured or dead cells reacted to these dyes by diffuse staining of the entire cell, including the nucleus. In 1917, Pappenheimer[3] made suspensions of rat thymus and human tonsils to study lymphocytes in vitro, and used a solution of trypan blue to stain the cells injured by various agents. He studied the effect of pH, heat, and anaerobiosis on lymphocytes. Schrek[4] modified Pappenheimer's method and studied the effect of moccasin venom, ricin, and temperature on a cellular suspension of Walker carcinoma 256. By this method, studies were also made on the effect of high temperature and moccasin venom,[5] x-rays,[6, 7] nitrogen mustard,[8] antiseptics,[9] local anesthetics,[10] adrenal cortex hormones,[11, 12] glucose,[13] and an oxidation-reduction indicator.[14]

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Received for publication, Mar. 17, 1953.

METHODS

With aseptic technique the tumor was resected, thoroughly minced with small sharp scissors, and filtered through an 80-gauge Monel wire screen. Human serum was added as needed during the mincing and filtering process. A suspension of isolated cells was thus obtained. To 0.4 ml. of the suspension was added 3.6 ml. of 1:4000 safranin in Tyrode's solution. A drop of the mixture was transferred to a hemocytometer, and the unstained cells were counted. The unstained cells were assumed to be viable. Stained cells were considered dying or dead, and were not counted. The suspensions used had counts of 25,000 to 50,000 viable cells per cubic millimeter. After the tumor cell suspension was prepared, appropriate dilutions of the cancerocidal agent were added to the suspension. All reagents and preparations were tested for sterility by use of agar plates.

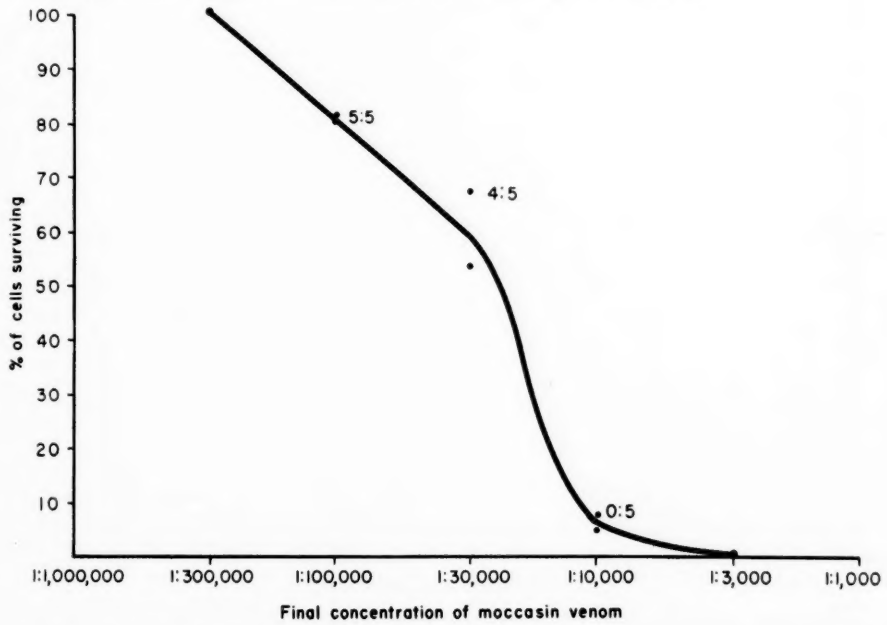

Fig. 1.—Effect of moccasin venom and incubation at 37° C. for twenty-one hours on survival of tumor cells and on number of successful transplants in rats.

Abscissa shows logarithm of the final concentration of venom in the cellular suspension. Ordinates represent the percentage of surviving cells with 100 per cent representing the number of cells surviving at 37° C. for twenty-one hours in the control suspension. The ratios in the graph show the number of takes of tumor in rats; the denominator indicating number of rats inoculated, and the numerator the number of rats developing tumor.

The preparations were distributed in 0.4 ml. amounts in 100 by 13 mm. test tubes and were placed in a water bath thermostatically regulated to 37° C. and equipped with a shaker (200 cycles per minute). Viable cell counts were made on the treated and untreated suspensions both before and after incubation. Two counts were made on each tube, and two tubes were counted for each dilution.

Following completion of the counts, the preparations were removed from the water bath, and the contents of each series of tubes pooled. Five rats for each suspension were injected subcutaneously with 0.1 ml. of suspension. The rats were inspected every two or three days and the tumors were measured in length, width, and depth. The rats without tumors were discarded one month following injection.

Special procedures were needed for handling the various reagents. The dry, adrenal cortex extract was quantitatively dissolved in methyl alcohol. Aliquot portions were distributed into sterile test tubes, and then vacuum dried at room temperature. By this pro-

cedure each test tube contained a known quantity of dry extract. The dried, powdered venom was dissolved in sufficient sterile distilled water to make a 1:100 solution and sterilized by passing through a Seitz filter. Intradermal tests in the rabbit showed that the minimal concentration of venom resulting in a red wheal was 1:8000 dilution. Stronger concentrations caused a definite intracutaneous hemorrhage.

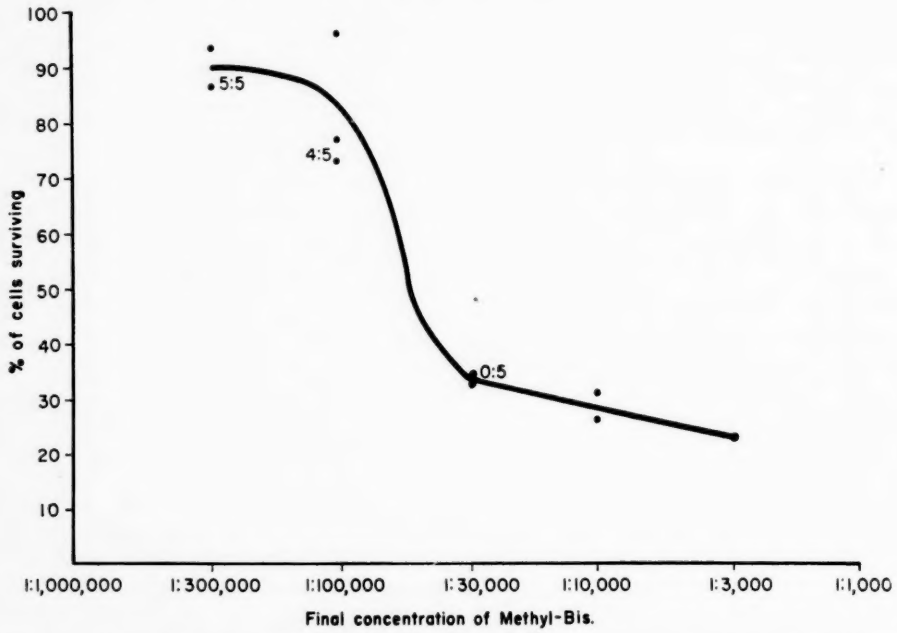

Fig. 2.—Effect of nitrogen mustard and incubation at 37° C. for twenty-two hours on survival of tumor cells and on number of successful transplants in rats.

Abscissa shows logarithm of the final concentration of methylbis (β-chloroethyl) amine hydrochloride in the cellular suspension. Ordinates and the ratios in the graph are described in Fig. 1.

RESULTS

Moccasin Venom.—The effect of moccasin venom and incubation at 37° C. for twenty-one hours is shown in the semilogarithmic curve of Fig. 1. The abscissa represents the logarithm of the final concentration of venom. The ordinate is the percentage of cells surviving, with 100 per cent representing the number of cells surviving at 37° C. for twenty-one hours in the control suspension without venom. The results of two experiments as shown in the graph seem consistent and can be represented by a single, S-shaped curve which is the usual type of curve obtained in dose-effect graphs. A dosage of 1:300,000 had no appreciable effect. A dose of 1:10,000 had practically a 100 per cent effect with nearly all the cells dead as a result of the venom. Between 1:100,000 and 1:30,000, the effect was incomplete, and the 50 per cent effect was produced by approximately 1:25,000 venom.

In one experiment, the results obtained on the effect of moccasin venom by the method of unstained cell counts were checked by inoculation of each incubated mixture into rats. One-tenth milliliter of each mixture of tumor cell suspension and venom was inoculated into each of five rats to observe tumor growth. The results are summarized in Fig. 1. It can be seen in the

figure that 100 per cent takes were observed in all rats injected with 0.1 and 0.01 ml. of the control suspension and with 0.1 ml. of the mixture containing 1:100,000 concentration of venom. Only one rat of five injected with cells in the presence of 1:30,000 concentration of venom did not develop a tumor. There were no takes in the 1:10,000 concentration. Both the unstained cell counts and the rat inoculations indicated that the minimum toxicity of moccasin venom was 1:30,000.

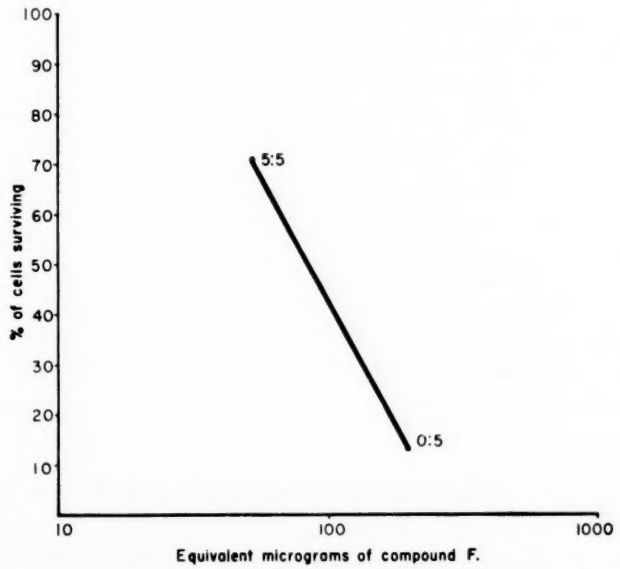

Fig. 3.—Effect of adrenal cortex extract and incubation at 37° C. for twenty-three hours on survival of tumor cells and on number of successful transplants into rats.

Abscissa shows logarithm of the equivalent micrograms of compound F per milliliter of cellular suspension.

Nitrogen Mustard.—Three experiments using methylbis (β-chloroethyl) amine hydrochloride (HN_2) were performed. The initial counts revealed a slight immediate toxic effect (26 per cent in the highest concentration 1:3000). The twenty-two hour results in vitro are represented by Fig. 2. The curve is S-shaped as with moccasin venom. The dose of 1:300,000 caused a slight effect which may be significant. A definite effect in two of three experiments was obtained with 1:100,000, and this might be considered the minimum effective dose. The 50 per cent dose is estimated to be 1:50,000 for nitrogen mustard as compared to 1:25,000 for moccasin venom. The maximum dose tested, 1:3000, permitted the survival of 23 per cent of the cells. This might be contrasted with the previous results obtained with venom where 1:3000 concentration allowed only 0.2 per cent of the cells to survive.

The effect of inoculation of the mixtures into rats is also summarized in Fig. 2. All rats inoculated with 0.1 and 0.01 ml. of control suspension developed tumors in less than six days. None of the rats injected with the cellular suspension treated with 1:30,000 nitrogen mustard developed a tumor, and only 4 of 5 successful takes were obtained in the presence of 1:100,000 reagent.

Where the cell suspension was treated with 1:300,000, all 5 rats developed tumors, with an average latent period of ten days. It is noted that the viable cells injected from the suspension treated with 1:30,000 nitrogen mustard numbered 600,000 cells with no takes, while the diluted control numbered only 130,000 with 100 per cent takes. These findings suggest that the nitrogen mustard not only killed the tumor cells but also may have inhibited slightly the growth of the surviving cells. Both the unstained cell counts and the inoculations into rats showed that a dose of 1:100,000 nitrogen mustard produced a minimal toxic effect to the tumor cells in the test tube.

Adrenal Cortex Extract.—A relatively high concentration of adrenal cortex extract (200 microgram equivalents of compound F per milliliter) showed a toxic effect by the unstained cell count method (Fig. 3). There was no demonstrable initial effect, but a definite delayed effect (2,500 viable cells as compared with 17,000 in the control). None of the 5 rats in this group developed a tumor in thirty-two days at which time the experiment was terminated.

The suspension containing 50 μg equivalents of compound F per milliliter showed a slight effect in twenty-three hours (12,000 cells per cubic millimeter with the reagent as compared to 17,000 cells per cubic millimeter with the control), as measured by the unstained cell count method, and all 5 rats developed a tumor with a delayed average latent period of eleven days. This experiment indicated a slight toxic effect of 50 μg equivalents of adrenal cortex extract on the tumor cells in suspension according to the unstained cell counts and the injection into rats.

Radioactive colloidal gold (Au^{198}): Two experiments were performed using Au^{198} as the test agent (Fig. 4). Unfortunately, all four dilations of Au^{198} in the first experiment were bacterially contaminated during the procedure, and the results thereof must be considered invalid. In the second experiment inactive gold in dilutions of 0.05 and 0.025 ml. of colloidal gold per milliliter had no appreciable effect by either the unstained cell count method or the inoculation into rats. With 100 microcuries of radioactive gold, no toxicity was discernible by the unstained cell count method, but the radioactivity did prevent tumor growth in the 5 rats inoculated with the treated tumor cells.

On the basis of these experiments it may be concluded that radioactive gold (Au^{198}) had an effect on tumor cells (apparently inhibition of mitosis). This deleterious effect was not indicated by the unstained cell count method, but was demonstrated by injection into susceptible animals.

DISCUSSION

Toxicity of Reagents.—The findings obtained on the toxicity of the reagents on the cells of the Walker tumor are summarized in Table I which shows estimations of the 50 per cent cytotoxic dose. As indicated previously, varying amounts of reagent were incubated with the cellular suspension at 37° C. for twenty-two hours. Viable cell counts were then made and rats were inoculated with the incubated, treated suspensions. The 50 per cent cytotoxic dose was the amount of reagent that reduced the viable cell count to one-half of that of the control incubated suspension or the dosage that

allowed tumor takes in only 50 per cent of the rats. It should be noted that the 50 per cent dosages determined in this study were only crude estimations to illustrate the method and not to determine the toxicity of the agents. Table I shows the correlation between the 50 per cent lethal doses as determined by unstained cell counts and by rat inoculations.

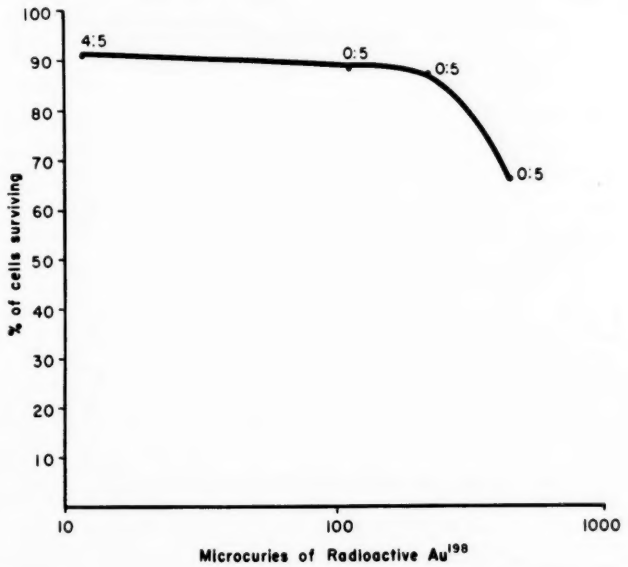

Fig. 4.—Effect of radioactive gold and incubation at 37° C. for twenty-two hours on survival of tumor cells and on number of successful transplants into rats.
Abscissa shows logarithm of the microcuries radioactive gold (Au^{198}) per milliliter of cellular suspension.

It is seen in the table that by unstained cell counts, the 50 per cent dose to the tumor cells was about 50 μg per milliliter for moccasin venom, 20 μg for nitrogen mustard, and 70 μg equivalents of compound F for adrenal cortex extract. The 50 per cent lethal doses according to rat inoculations were approximately the same as that determined by unstained cell counts. For radioactive gold the 50 per cent lethal dose was small (50 microcuries per milliliter) according to the rat test and large (more than 565) according to the in vitro counts. It seems then there was good correlation of the two methods in evaluating the cytotoxicity of the first three reagents, but was poor with radioactive gold. Evidently a reagent may have toxic properties which are not demonstrable by the method of unstained cell counts.

The in vitro test was able, then, to determine the direct cytocidal action of the reagent to the tumor cells. The question now arises what is the toxicity of the agents to normal cells. Theoretically, one should use as a control the type of cells from which the tumor originated. But it is usually not essential to use the same types of normal and cancer cells in testing a chemotherapeutic agent. Ultimately, the chemotherapeutic agent would be expected to have a greater effect on tumor cells than on all the normal cells in the host. Therefore it may be feasible to use as a control another type of normal cell,

TABLE I. ESTIMATIONS OF THE 50 PER CENT CYTOTOXIC DOSE OF FOUR REAGENTS TO CELLS OF THE WALKER RAT TUMOR 256 AND TO RAT THYMOCYTES ACCORDING TO UNSTAINED CELL COUNTS AND ACCORDING TO INOCULATIONS OF THE TREATED TUMOR CELLS INTO RATS

REAGENT	50 PER CENT CYTOTOXIC DOSE		
	CELLS OF WALKER RAT TUMOR 256		RAT THYMOCYTES
	UNSTAINED CELL COUNTS	RAT INOCULATIONS	UNSTAINED CELL COUNTS
Moccasin venom (μg per ml.)	50	50	50
Nitrogen mustard (μg per ml.)	20	20	2
Adrenal cortex extract (μg equivalents of compound F per ml.)	70	100	0.1
Radioactive gold (microcuries per ml.)	>565	50	20

particularly one that is sensitive to toxic agents. The thymocyte seems to be a useful cell as a test object to serve as a control to all types of tumor cells in in vitro chemotherapeutic studies. Any agent found to be much more toxic to tumor cells than to the thymocyte would, we believe, be worthy of a careful test in vivo.

In view of the previous work from this laboratory with the method of unstained cell counts on normal cells,[4-9] the present paper stresses chiefly the reactions of tumor cells to the reagents. It may, however, be of interest to include some previously published and unpublished findings on the effect of the reagents on the rat thymocyte. The thymocytes were suspended in 50 per cent rat serum, although the tumor cells were tested in 50 per cent human serum.

Table I shows that moccasin venom had approximately the same toxicity to the malignant and the normal cells. It is seen, furthermore, that the rat lymphocytes were more sensitive to nitrogen mustard, adrenal cortex extract, and to radioactive gold than the tumor cells. According to the results presented in the table, none of the four reagents would be considered to have a specific cancerocidal action, i.e., a greater toxicity to the Walker tumor cells than to the normal thymocytes.

Screening of Reagents.—This paper offers the method of unstained cell counts as a means of screening agents for cancerocidal activities. This proposal is based on the following assumptions or hypotheses: first, there is an agent or agents that can inhibit or destroy human cancer. Second, this hypothetical agent is more toxic or lethal to human tumor cells than to normal cells. Third, it is also more toxic to animal tumor cells (such as the Walker carcinoma 256) than to normal ones (as rat lymphocytes). Finally, this differential toxicity can be demonstrated in vitro by the method of unstained cell counts.

It is to be noted that these or other assumptions are expressed or implied in any screening program—whether in vivo or in vitro. The only valid proof of any set of assumptions or any method of screening would be the finding of an agent against human cancer. Until such a finding, no screening method can be considered as proved, as standard, or as possessing greater validity or usefulness than another.

The question still remains whether the assumptions for the present in vitro methods are reasonable. Is it reasonable to expect that the cells would

react in vitro under artifical conditions as they react in vivo? Let us see whether the in vitro method has in the past yielded results which were in accord with in vivo findings.

The method of unstained cell counts has been used to study the effect of x-rays on lymphocytes in vitro and has led to the following findings: first, x-rays had a direct but delayed (three or more hours) cytocidal action on lymphocytes (Schrek[6] and Patt, Blackford, and Straube[15]). Second, intranuclear vacuoles developed in living lymphocytes, three hours after irradiation, and the vacuoles killed the cells in twenty minutes (Schrek[7, 16]). Third, lymphocytes were not affected by x-rays when the cells were incubated at 17° C. instead of at 37° C. (Schrek[6]).

These findings may be correlated with several in vivo observations. First, Ross, Furth, and Bigelow[17] found that four to eight hours after the irradiation of rats, many injured and dead lymphocytes appeared in the thoracic duct. Second, intranuclear vacuoles were observed by Warren, Holt, and Sommers[18] in tissues of rats and mice irradiated in vivo. The vacuoles caused the death of the radiosensitive lymphocytes but did not kill the radioresistant liver cells. And third, Allen, Schjeide, and Hochwald[19] reported that x-irradiation killed tadpoles kept at room temperature but did not affect them at 17° C. or below. These investigators remarked, "Thus his (Schrek's) results and our own are in good correspondence. This point is quite interesting in that there is involved a comparison between cold-blooded and a warm-blooded animal, also between in vitro and in vivo treatment."

Evidently the in vitro and in vivo findings on the action of x-rays on lymphocytes are in accord not only qualitatively but also—according to several indications—quantitatively. The in vitro method has yielded informative data on the radiosensitivity of lymphocytes and should be able to provide data on the toxicity of agents to cancer and normal cells.

Our conclusions are that the method of unstained cell counts is a reasonable means of screening cancerocidal agents. Furthermore the method is simple, rapid, reproducible, and relatively inexpensive and provides quantitative results. The in vitro test seems to be a means of screening agents for inclusion among those to be given an in vivo trial rather than a method for excluding agents for further consideration. It should prove useful in the rapid screening of large numbers of chemically related compounds of varying cancerocidal potential.

SUMMARY

The in vitro method of unstained cell counts is proposed as a means of screening the cytocidal and the cancerocidal activity of reagents. This procedure was tested by determining the cytocidal action of 4 reagents against the cells of Walker tumor 256 and against rat thymocytes. The reagents in varying amounts were incubated with the tumor and normal cells at 37° C. for twenty-two hours. The number of viable cells was then determined by adding a safranine solution and counting the unstained cells. The reagents tested were moccasin venom, nitrogen mustard, adrenal cortex extract, and radioactive gold. All of the reagents had the same or a lesser toxic action

on the tumor than the normal cells. According to the method of unstained cell counts, none of the reagents had apparently any specific cancerocidal activity.

The findings were further controlled by inoculating the incubated mixtures of reagent and tumor cell suspensions into susceptible animals and observing the number of takes and the latent periods of the tumors. The findings obtained by unstained cell counts and by rat inoculations were in good agreement on the toxicity of three of the reagents to the tumor cells. Radioactive gold had, however, a low toxicity according to the method of unstained cell counts, but the reagent did prevent the growth of the tumor cells on inoculation into rats. Apparently Au^{198} was antimitotic but not cytocidal.

The unstained cell counts seemed to be a simple in vitro procedure for obtaining data on the relative cytocidal action of agents to normal and tumor cells.

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